The effect of the polyadenylation inhibitor Cordycepin on MCF-7 cells

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Declaration

Except where acknowledged in the text, I declare that this dissertation is my own work and is based on research that was undertaken by me in the School of Pharmacy, Faculty of Science, University of Nottingham.

Abstract

Cordycepin (3'-deoxyadenosine) is a medicinal bioactive component of the caterpillar fungi (*Cordyceps* and *Ophicordyceps*). It is reported to have nephroprotective, antiapoptotic, anti-metastatic, hepatoprotective (Yue et al. 2013), inflammatory effects, antioxidant, anti-tumor, immunomodulatory and vasorelaxation activities. Cordycepin is well known to terminate and inhibit polyadenylation, both *in vitro* and *in vivo*. Other proposed mechanisms of action of cordycepin include activation of adenosine receptors, activation of AMP dependent kinase (AMPK) and inhibition of PARP1.

The purpose of this study is to elucidate the biological and pharmacological effects of cordycepin on cancer cell lines such as MCF-7 cells. In this study I found that cordycepin reduces the cell proliferation in all examined cell lines without always exerting an effect on 4EBP phosphorylation and protein synthesis rates. Therefore, the effects on protein synthesis via inhibition of mTOR, which were previously reported, are not only the sole reason for the effect of cordycepin on cell proliferation. Knockdown of poly (A) polymerases reduces cell proliferation and survival, indicating that poly (A) polymerases are potential targets of cordycepin. I studied different adenosine analogues and found that 8 aminoadenosine, the only one that also consistently inhibits polyadenylation, also reduces levels of P-4EBP. It also inhibits the expression of specific genes indicating that the effects on polyadenylation, mTOR signalling and gene expression are linked. Also consistent with polyadenylation inhibition as the major mode of action is the fact that the effects of cordycepin on gene expression are predominantly post-transcriptional. However, knockdown of poly (A) polymerases did not have the same effects on gene expression or on polyadenylation, indicating that cordycepin may act as a dominant negative rather than as a null mutant. This is consistent with the fact that cordycepin is known to arrest a normally transient polyadenylation complex.

We performed microarray analysis of cordycepin treated MCF-7 cells and found that the downregulated mRNAs were predominantly involved in transcriptional regulation, cell proliferation, cell cycle and cell migration. These data show that

cordycepin is a promising new drug for cancer and indicates that the mode of action it is likely to be through the inhibition of polyadenylation.

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4E-BP	eIF4E-binding proteins
18S rRNA	18S ribosomal RNA
3' UTR	3' untranslated region
^{35}S	Sulphur-35
5'-[^{32}P]pCp	Cytidine-3',5'-bis-phosphate
A549	Human alveolar adenocarcinoma cell line
ACTB	Actin, beta
Ado	Adenosine
ADP	Adenosine diphosphate
AGTR2	Angiotensin II receptor, type 2
AICAR	5-aminoimidazole 4-carboxamide riboside
AMPK	Adenosine monophosphate dependent protein kinase
APA	Alternative cleavage and polyadenylation
APS	Ammonium persulphate
ARE	AU rich element
ASM	Primary human airway smooth muscle cells
ATF4	Activating transcription factor 4
ATP	Adenosine 5'- triphosphate
AUF1	AU-rich binding factor 1
BRCA1	Breast cancer type 1 susceptibility protein
BRF1	Butyrate response factor 1
BSA	Bovine Serum Albumin
BTG1	B-cell translocation gene 1
CASP8	Caspase 8, apoptosis-related cysteine protease
CATSPER2	Cation channel, sperm associated 2
CBFB	Core-binding factor subunit beta
CCL2	Chemokine (C-C motif) ligand 2
CCR4	Carbon catabolite repression 4
CD36	Thrombospondin receptor
CDH1	Cadherin-1
Cdk	Cyclin dependent kinase
CDKA	Cyclin dependent kinase A
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
CF I	Cleavage factor I
CF II	Cleavage factor II
CK	Cytokeratin

CM	Cordyceps Militaris
CNOT4	CCR4-NOT transcription complex, subunit 4
COX2 (PTGS2)	Cyclooxygenase
CPE	Cytoplasmic Polyadenylation Element
CPE	Carboxypeptidase E
CPEB	Cytoplasmic Polyadenylation Element Binding Protein
CPSF	Cleavage and Polyadenylation Specificity Factor
CS	Cordyceps Sinensis
CstF1	Cleavage stimulation factor, 3' pre-RNA, subunit 1
CTD	C terminal domain
CTSL1	Cathepsin L1
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL8 or (IL8)	Interleukin 8
dATP	Deoxyadenosine 5’-tripohosphate
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DCP1A	Decapping enzyme homolog A
dCTP	Deoxycytidine 5’-triphosphate
DDR	DNA damage response
Deptor	DEP-domain containing mTOR-interacting protein
dGTP	Deoxyguanosine 5’-triphosphate
DMEM	Dulbecco’s Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide 5’-triphosphate
DRB	5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole
DSE	Downstream sequence elements
DSIF	DRB- sensitivity inducing factor
dsRNA	Double stranded RNA
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine Tetraacetic Acid
eEF2	Eukaryotic translation elongation factor 2
eEF2K	Eukaryotic translation elongation factor 2 kinase
EGR1	Early growth response 1
EGTA	Ethylene glycol tetraacetic acid
eIF (4A,4E,4G)	Eukaryotic translation initiation factors (4A,4E,4G)
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ErbB	Erythroblastic leukemia viral oncogene
ERK	Extracellular signal-regulated kinase

ERRB2	ERRB2 estrogen-related receptor beta-2
FAT	FAT-carboxy terminal domain
FATC	FRAP-ATM-TTRAP domain
FBS	Foetal Bovine serum
FBXW7	F-box and WD repeat domain containing 7
FCS	Foetal calf serum
FOXA1	Forkhead box protein A1
FRB	FKBP12-rapamycin binding domain
GAB2	Grb2-associated binding protein 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3	Trans-acting T-cell-specific transcription factor/GATA-binding factor 3
GNAI3	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3
GRB7	Growth factor receptor-bound protein 7
GTFs	General transcription fcators
GTP	Guanosine triphosphate
HCl	Hydrogen chloride
HCT116	Human colorectal carcinoma cells
HEAT	Huntingtin-Elongation factor 3-regulatory subunit A of PP2A-*TOR1*
Hela	Human cervical epithelial carcinoma cells
Hepes	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulphonic Acid
HER2	Human epidermal growth factor receptor 2
HIF1A	Hypoxia inducible factor 1, alpha subunit
HRP	Horseradish peroxidase
HT29	Human colorectal adenocarcinoma cells
IGF-1R	Insulin-like growth factor receptor 1
IHC	Immunohistochemistry
IL6	Interleukin 6
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
kDa	Kilodalton
KHSRP	KH-type splicing regulatory protein
KRAS	Kirsten rat sarcoma viral oncogene
LDLRAP1	Low density lipoprotein receptor adaptor protein 1
LIF	Leukemia inhibitory factor
LKB1	Liver kinase B1
MAP3K1	Mitogen-activated protein kinase kinase kinase 1 or MAPK/ERK kinase kinase 1
MAP3K13	Mitogen-activated protein kinase kinase kinase 13

MAPK	Mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation - 7
MCL1	Myeloid cell leukemia sequence 1
MED13L	Mediator complex subunit 13-like
MED26	Mediator complex subunit 26
MED9	Mediator complex subunit 9
MEK	Raf/mitogen-activated and extracellular signal-regulated kinase kinase
MEK1/2	Map or ERK kinase 1 or 2
$MgCl_2$	Magnesium chloride
miRISC	miRNA induced silencing complex
miRNA	MicroRNA
mLST8	Mammalian lethal with SEC Thirteen 8
mRNA	Messenger ribonucleic acid
mSIN1	Mammalian stress-activated protein kinase interacting protein 1
MTA	5'-Deoxy-5'-(methylthio)adenosine
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MTPAP (papd1)	Mitochondrial poly(A) polymerase
MYC	v-myc avian myelocytomatosis viral oncogene homolog
MYH9	Myosin heavy chain 9
NaCL	Sodium chloride
NCOR1	Nuclear receptor corepressor 1
NEK6	NIMA (never in mitosis gene a) related kinase 6
NELF	Negative elongation factor
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NIH3T3	Primary mouse embryonic fibroblast cells "3-day transfer, inoculum 3 x 10^5 cells
Nt	Nucleotide
NTP	Nucleoside triphosphate
NUDT21	Nucleoside diphosphate linked moiety X)-type motif 21
P-4EBP	Phosphorylated-eIF4E-binding proteins
PABN1	Nuclear poly (A)- binding protein 1
PABP	Poly-A Binding Protein
PABPC	Cytoplasmic poly (A)- binding protein
PAGE	Polyacrylamide gel electrophoresis
PAIP	PABP interacting protein 1
PAN	Poly (A) nuclease
PAP	Poly(A) polymerase

PAPD2	PAP-associated domain-containing 2
PAPD4	PAP associated domain containing 4
PAPd5	PAP associated domain containing 5
PAPd7	PAP associated domain containing 7
PAPOLA	Poly(A) polymerase alpha
PAPOLB	Poly(A) polymerase beta
PAPOLG	Poly(A) polymerase gamma
PARN	Poly (A)- specific ribonuclease
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDCD4	Programmed cell death 4
PDK1	Phosphoinositide-dependent protein kinase 1
PHAS-I	Phosphorylated heat and stable protein, insulin stimulated
PI3K	Phosphoinositide 3-kinase
PIC	Preinitiation complex
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIK3CB	Phosphoinositide-3-kinase, catalytic, beta polypeptide
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide
PIP2	Phosphatidylinositol (4,5)-biphosphate
PIP3	PtdIns(3,4,5)P3), phosphatidylinositol (3,4,5)- trisphosphate
PLK2	Polo-like kinase 2
Pol II	RNA Polymerase II
PR/PgR	Progesterone receptor
PRAS40	Proline rich AKT substrate 40 kDa
Protor-1	Protein observed with Rictor-1
P-TEFb	Positive transcription elongation factor b
PTEN	Phosphatase and tensin homolog or phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase
PTN	Pleiotrophin
q-PCR	quantitative real time polymerase chain reaction
Raptor	Regulatory associated protein of mTOR
RB1	Retinoblastoma 1
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive component of mTOR
RN18S	18S ribosomal RNA
RNA	Ribonucleic acid
RNase	Ribonucleic acid hydrolase
RNPS	Ribo-nucleo proteins
RPL10A	Ribosomal protein L10a
RPs	Ribosomal proteins

rRNA	Ribosomal RNA
RUNX1	Runt-related transcription factor 1
S6K1	Ribosomal S6 protein kinase 1
SDS	Sodium Dodecyl Sulphate
Se13	Secret 13 compund
SF3B1	Splicing factor 3b, subunit 1
siRNA	Silencing ribonucleic acid
SMARCD1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1
SNAI3	Snail family zinc finger 3
ssRNA	Single stranded RNA
STMN2	Stathmin-2
SUN1	Sad1 and UNC84 domain containing 1
TAE	Tris acetic acid EDTA buffer
TBE	Tris-borate EDTA buffer
TBS	Tris buffered saline
TBST	Tris Buffered Saline Tween
TBX3	T-box transcription factor
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
TFPI	Tissue factor pathway inhibitor
TKIs	Tyrosine kinase inhibitors
TOS	TOR signaling motif
TP53	Tumor protein p53
Trf4	Topoisomerase I-related protein
tRNA	Transfer RNA
TSC1 and TSC2	Tuberous sclerosis complex 1 and 2
TTP	Tristetraproline
uORF	Upstream open reading frame
USE	Upstream sequence elements
UTR	Untranslated Region
ZFP36	Zinc finger protein 36, C3H type
ZFP36L1	Zinc finger protein 36, C3H type-like 1

1 Introduction

1.1 Breast cancer

Approximately 13% of all worldwide deaths in 2008 are related to different types of cancer, representing 7.6 million deaths (Gutschner and Diederichs 2012). In general cancer affects 1 in 3 people in the duration of their lifetime (Kaye 1999). In USA the average age of breast cancer identification is 61 years (Roskoski 2014). Known risk factors for breast cancer include smoking, tobacco, infections, excessive weight gain and radiation. Breast cancer is the development of abnormal cells that keep growing beyond the normal boundaries of tissue repair (Hanahan and Weinberg 2011). Somatic alterations in human genome are involved in developing cancer such as translocations, single base substitutions and copy number alterations. Investigation of these alterations provides insights into the cellular defects (Beroukhim et al. 2010). Among women, breast cancer is the most commonly diagnosed form of cancer especially in USA and Europe and the main cause of death related to cancer worldwide (Eroles et al. 2012). In 2008, 182,640 women and 1,990 men were diagnosed with breast cancer (Meacham et al. 2009).

1.1.1 Molecular subtypes of breast cancer

Breast cancer is a heterogeneous, complicated and diversified disease rather than a simple single disease. It comprises a mixture of various subtypes, which were first analyzed by high throughput gene expression profile by Perou in 2000 into four subtypes and a year later further divided into luminal subtypes A and B by Sorlie in 2001 (Perou et al. 2000, Sasaki et al. 2013). Breast cancer can be divided into six molecular subtypes: HER2-enriched (hormone receptor negative/HER2 positive), Basal- like (hormone receptor negative/HER2 negative), normal breast cancer (hormone receptor negative/HER2 negative), luminal A (hormone receptor positive/HER2 negative), luminal B (hormone receptor /HER2 positive) and claudin-low (hormone receptor/HER2 negative). All are linked with distinct prognosis (Eroles et al. 2012, Previati et al. 2013).

Normal and basal subtypes of breast cancer are negative for all three key receptors (ER, PgR, HER2) but they differ in expression pattern of CK5 and EGFR which are positive for basal-like while negative for normal subtype. The claudin-low subtype is also predominantly ER/PgR/HER2 negative but 20% of these tumours are hormone receptor positive, in contrast with basal-like which are 100% negative for ER/PgR/HER2 (Eroles et al. 2012). Basal-like and HER2 subtype are found to be the most commonly diagnosed breast tumours in young women. They are characterized by high levels of EGFR, a familiar marker of basal-like breast tumours, which are most agressive. The basal-like subtype of breast cancer, also known as triple negative, has been defined by immunohistochemistry. Characteristic features of molecular subtypes of breast cancer, based on their clinical and gene expression pattern, have been summarized in table 1. Both subtypes of luminal are defined by expression of hormone receptors gene. Luminal A posses greater levels of estrogen receptors compared to luminal B. The key marker of identification between these two subtypes relate to the high expression of KI67 and cyclin B proliferation genes (Eroles et al. 2012).

1.1.2 Role of receptors in Breast cancer

Breast cancer involves deregulation of the growth factor and hormone receptors that are also used to classify them. Key receptors of breast cancer are HER2, estrogen receptor and progesterone receptor used as a biomarker play crucial role in identification and treatment of various sub types and grades of breast cancer (Sekar et al. 2011).

1.1.2.1 HER2

Alterations in the HER2 receptor pathway result in a loss of maintenance of proliferative signalling in breast cancer. HER2 is an oncoprotein linked with subfamily of HER (human epidermal receptor) family and associated with EGFR (epidermal growth factor receptor) family. HER2 can formeither homo or heterodimers in the presence of ligand, which leads to activation of tyrosine kinase function inside the cell. 10% -30% of breast tumours have amplification of HER2(Cornejo et al. 2014). The signalling pathways which are mainly effected by

these receptors are PI3K/AKT/mTOR and Ras/Ref/MAPK. These pathways are major regulators of cell growth, proliferation and survival, metabolism, division and apoptosis (Eroles et al. 2012).

1.1.2.2 Hormone receptors

Steroid hormone receptors (estrogen receptor (ER) and progesterone receptor (PR)) interact with hormones and act in the nucleus. Progesterone receptors serve as an indicative marker in determining the status of estrogen levels. This helps to determine the choice and response of therapy among patients, as levels of ER serves as crucial role in PR expression. Breast cancer patients with progesterone receptor expression (PR^+) in their tumours have better rates of survival compared to those who are are PR^- (Cornejo et al. 2014).

Estrogen receptors are the major players of endocrine signaling. There are two isoforms: ERα and ERβ. ERα has a crucial role in advancement of breast cancers as around 70-75% of breast cancer are estrogen receptor α positive ($ER\alpha^+$) (Ciruelos Gil 2014). One of the ER^+ cell lines of breast cancer which is used extensively in research is MCF-7. Estrogen receptors bind to estrogen, a steroid hormone, which plays a critical role in the female reproductive cycle. The association of estrogen to its receptor not only affects the function of targeted genes, but also growth and differentiation. Estrogen receptors modulate gene expression by serving as a ligand mediated transcription factors. The binding of ligand stimulates changes in receptor conformation and results in dimer formation, DNA binding, addition of co-activator molecules in addition with other transcription factors and release of co-repressors. ERs transfer to the nucleus upon binding to estrogen, which enhances the expression of targeted genes either through the interaction of the ER with the ERE (estrogen response element) or by association with a co-activator molecule (Xu et al. 2013).

1.1.3 Breast cancer treatment

Hormone receptor positive subtypes of breast tumour, such as luminal A and B, are generally treated by endocrine therapy, as detailed below. In addition, chemotherapy is also required for the efficient treatment of luminal B subtype.

HER2 positive subtype (36%) and basal-like (43%) are highly sensitive to chemotherapy compared with luminal A (7%) and B (17%) (Eroles et al. 2012). For basal-like subtype of breast cancer, PARP1 inhibitors are used in combination with DNA damage inducing chemotherapeutic agents such as platinum salts (e.g: carboplatin, cisplatin) (Anders and Carey 2009). Effective treatments for each subtype of breast cancer are summarized in table1.

1.1.3.1 HER2^{+} treatment

The HER2^{+} subtypes of breast cancer are characterized by increased expression of the HER2 gene or the genes related to the HER2 pathway found on 17q12 chromosome. The breast cancer shows resistance towards endocrine therapy but are effectively cured by anti-HER2 treatment such as trastuzumab (Genentech, Inc., San Francisco) a monoclonal antibody and lapatinib (GlaxoSmithKline, King of Prussia, Pennsylvania). Anti-HER2 treatment is found to be more efficient when used in combination with chemotherapy, which reduces the relapse rate to 50% and enhances the survival rate to 33% (Cornejo et al. 2014).

1.1.3.2 Endocrine therapy

Endocrine therapy is a direct targeted therapy proposed for ER and PgR positive subtypes of breast tumour, which inhibits the growth enhancing effects of estrogen through estrogen receptors. The effective response length of endocrine therapy is 8-14 months for 20-40% ER positive patients. Types of endocrine therapy include selective estrogen receptor modulators (SERMS), such as tamoxifen, which block ligand binding to ER. Others regulate the transcription of ERs in a tissue specific manner, by downregulating the ER expression (such as fulvestrant), while some block the synthesis of estrogen such as analogs of gonadotropin-releasing hormone. Aromatase inhibitors (AI) inhibit the activity of the aromatase enzyme which converts androgens into estrogen (like letrozole, exemestane) (Ciruelos Gil 2014). Tamoxifen treatment is effective for most women independent of menopausal status, compared to AI which is more useful in post menopausal women. Patients with high levels of ERα respond better to

endocrine therapy compared to the patients with low to negligible levels of ERα, which react better to chemotherapy (Xu et al. 2013). Tumours in patients with high levels of HER2 have greater resistance towards SERMS compared to estrogen reduction therapy (AI) (Cornejo et al. 2014).

1.1.3.3 Chemotherapy

Chemotherapy is one of the most popular options for breast cancer treatment. It gives better outcomes with enhanced rates of disease-free survival especially when used in combination with endocrine therapy. Chemotherapy reduces mortality rate by 33% with 45% decline in recurrence rate when used in combination with endocrine therapy. The major limitation of chemotherapy for breast cancer treatment is drug resistance. For luminal B, $HER2^{+}$ and basal like subtypes of breast cancer, chemotherapy is preferred. The molecular mechanism behind chemotherapy resistance in ERα positive patients is still unknown. A reduced rate of proliferation in MCF-7 breast cancer lines has been reported, when chemotherapeutic agents (paclitaxel, 5-fluorouracil) are used in combination with endocrine therapy (AI and SERMS) (Xu et al. 2013).

Table 1: Tumour Biology of breast cancer

Molecular Subtypes	Receptors (ER/PR/HER2)	Frequency	Histological grade	Prognosis	Proliferation Genes (Ki67)	Molecular pathways	Relapse rate	Site of origin	Markers identified by IHC	Treatment
HER2 (or ERRB2) enriched	$ER^-/PR^-/HER2^+$	15-20%	high	bad	high	HER2 genes, mutations in TP53	_	Epithelium of breast duct	HER2, GRB7	Chemotherapy ,anti-$HER2^+$ therapy
Basal-like OR Triple negative (TN)	$ER^-/PR^-/HER2^-$	10-20%	high (gradeIII/ highly aggressive)	bad	high	High rate of TP53 mutations (44%), BRCA1	Initial 3 years	Myoepithelial breast cells	$CK5^+$, $CK6^+$, $CK17^+$,$CK14^+$,$EGFR^+$ ($HER1^+$),$CD44^+$, caveolin 1^+, P-$cadherin^+$	Chemotherapy , PARP inhibitors, cytotoxic agents e.g platinum salts
Normal-like	$ER^-/PR^-/HER2^-$	5-10%	low	middle	low	_	_	Adipose tissue	PTN, CD36 $EGFR^-$, $CK5^-$	Poor response to chemotherapy , U-I
Luminal A	$ER^{3+}/PR^{3+}/HER2^-$	50-60%	low	good	low	ER sensitive genes	27.8%	Luminal epithelium of mammary ducts	$CK8^+$, $CK18^+$, $GATA3^+$	Endocrine therapy
Luminal B	$ER^+/PR^{+/-}/HER2^{-/+}$	10-20%	intermediate	Bad/ middle	high	ER sensitive genes, mutations in TP53	1.6 years	Luminal epithelium of mammary ducts	$CK8^+$, $CK18^+$, $GATA3^+$	Endocrine therapy, chemotherapy
Claudin low	$ER^-/PR^-/HER2^-$	12-14%	high	bad	high	_	_	_	SNAI3	Poor response to chemotherapy , U-I

Abbreviations: CK: cytokeratin, PR: progesterone receptor, ER: estrogen receptor, GATA3: GATA binding protein 3, HER2: human epithermal receptor protein-2, IHC: immunohistochemistry, CD36: thrombospondin receptor, GRB7: growth factor receptor-bound protein 7, SNAI3: snail family zinc finger 3, PTN: pleiotrophin, ERRB2: estrogen-related receptor beta-2, U-I: under-investigation

Adapted from: (Earp et al. 2003, Anders and Carey 2009, Eroles et al. 2012, Cornejo et al. 2014).

1.1.4 Molecular mechanisms of breast cancer

The cellular and molecular pathways in the development of breast cancer involve several important mechanisms, such as the proliferative response of breast cells toward several peptide growth factors or involvement of the estrogen receptor (ER) and IGF (Insulin-like growth factor) pathways. IGF was found to play an important role in breast cancer progression by transforming normal cells to acquire tumorigenic characteristics, which consist of a chain of several components. These include insulin, IGF-1, IGF-2 and IGF-1 receptor (IGF1R), which are involved in mediating growth, metabolic and nutritional processes of cells (Oh et al. 2008, Werner and Bruchim 2012). IGF-IR exerts its action as an inhibitor of apoptosis and regulates the activities of insulin and IGF (IGF-1, IGF-2) (Jalving et al. 2010). High levels of IGF1R expression are found in aggressive types of breast cancer compared to benign tumor oruntransformed breast epithelial cells (Lee et al. 1998, Werner and Bruchim 2012).

Inactivating mutations in BRCA1 and BRCA2 have a pronounced effect on breast cancer development (King et al. 2003). BRCA1 genes are often known as ‘caretakers of the genome’ and play a vital role in identifying DNA damage and mediating DNA repair processes (Anders and Carey 2009). Under normal conditions BRCA1 and BRCA2 are involved in the DNA repair mechanism by homologous recombination (Earp et al. 2003). In breast cancer, inactive BRCA1 or BRCA2 leads to the collection of DNA strand breaks, which aids the growth of tumours. Cancerous cells with mutated BRCA1 or BRCA2 genes are more sensitive to DNA damage agents in combination with the drugs that inhibit the activity of enzymes involved in DNA damage repair, such as PARP (Poly (ADP-ribose) polymerase) (Vogelstein et al. 2013). PARP plays crucial role in repairing DNA single strands break after DNA

damage, while its lack of activity leads to collections of double strand DNA breaks, causing cell death. Therefore PARP inhibitors are used extensively in treating BRCA deficient cancer (Anders and Carey 2009, Frizzell and Kraus 2009, Kim et al. 2011).

1.1.5 Breast cancer hallmarks

There are some basic hallmarks of breast cancers (Hanahan and Weinberg 2000) which normal cells have to acquire in several stages in order to become tumorigenic. These are as follows:

1.1.5.1 Sustaining proliferative signaling

In general, normal or healthy cells manage to control the balance between growth inhibiting and promoting signals. In contrast, cancerous cells continue to proliferate endlessly without the need of any stimuli, as the cells become independent of growth signals and often have deregulated signaling pathways. Oncogenic mutations that enhance growth factor signaling include the activating mutations in the RAS and PI3K (PIK3CA) oncogenes and inactivation of the PTEN tumour suppressor. These activating mutations lead to growth factor signaling in the absence of growth factors. In other cases, mutations or overexpression of transcription factors that are normally induced by growth factors, such as FOS and MYC, leads to reduced dependence on growth factors for proliferation. Many novel cancer drugs target the growth factor signaling, but feedback regulation can lead to drug resistance. An example of such type of feedback mechanism involved in cancer is the mTOR pathway, which plays important roles in cell metabolism and growth. Activated mTOR inhibits PI3K signaling through negative feedback. Therefore, after inhibition of mTOR, PI3K signaling frequently is increased. This unltimately reduces the antiproliferative properties of inhibitors of this pathway (Hanahan and Weinberg 2011).

1.1.5.2 Evading growth suppressors

Tumor cells also have an ability to maintain proliferative signals by escaping from growth inhibiting factors. These factors normally induce several tumor suppressor genes and lead to apoptosis or an arrest in cell cycle progression. Inactivation of tumour suppressor genes such as Tp53 and Rb, either by mutation or by gene

regulation, therefore play a critical role in tumour progression. More than 50% of all human cancer has mutated Tp53 and in individuals where only one functional copy of the Tp53 is inherited, there is a higher chance of cancer (Gutschner and Diederichs 2012).

1.1.5.3 Capable of replicative immortality

Unlike normal cells in which the number of cell divisions is restricted, cancerous cells have a potential to replicate endlessly. In normal cells, senescence or cell cycle arrest limits the replicative potential. This may be due to DNA damage or the shortening of telomere lengthening enzyme telomerase after each cycle of cell division (Gutschner and Diederichs 2012). As cancerous cells become immortalized, about 90% of human tumor expresses the telomere lengthening enzyme telomerase at high levels. This type of DNA polymerase is responsible for adding telomeric hexanucleotide repeats at the end of each chromosome. The remaining 10% of tumor cells undergo ALT (alternative lengthening of telomeres) which involves the transfer of hexanucleotide repeats among chromatids (Gutschner and Diederichs 2012).

1.1.5.4 Activating invasion and metastasis

The major characteristic of cancer is its ability to spread or metastasize to other parts of a body. Most solid tumours are carcinomas, which arise from epithelial cells. Metastasized carcinomas are oftenmore aggressive and higher grade than the original tumour. They metastasize rapidly to distant organs by developing changes in their shape and their connections to ECM (extracellular matrix) and to other cells in a process, which is called the epithelial to mesenchymal transition (EMT) (Hanahan and Weinberg 2000, Gutschner and Diederichs 2012). Carcinoma cells lose a key cell matrix interacting molecule known as E-cadherin, which plays a crucial role in assembling epithelial cell layers by providing cell to cell adhesion support. Over-expression of E-cadherin blocks metastasis and invasion, therefore in cancerous cells, the expression of E-cadherin found to be downregulated (Gutschner and Diederichs 2012). The gene expression changes associated with the EMT also inhibit programmed cell death (Hanahan and Weinberg 2011).

1.1.5.5 Inducing angiogenesis

Cancerous cells like normal cells also need oxygen and nutrients and need to excrete carbon dioxide and metabolic waste to proliferate. The process of angiogenesis and vasculogenesis (formation of new endothelial cells and their assembly into a tube), normally happens during the process of embryogenesis, in adults during wound healing and in the female reproductive cycle. In normal tissues the process of vasculogenesis becomes inactivated once the tissue is fully formed. In tumors, angiogenesis and vasculogenesis remain active allowing continued proliferation of tumor cells (Hanahan and Weinberg 2011). One of the factors involved is VEGF-A (vascular endothelial growth factor-A) an angiogenesis activator which becomes activated upon oncogenic signals or hypoxia (Ferrara 2009, Gutschner and Diederichs 2012).

1.1.5.6 Resisting cell death

Cancer cells usually experience stresses which would normally induce cell death due to imbalanced signaling and damage to the DNA causing a high rate of replication (Hanahan and Weinberg 2000). Normal cells possess three main mechanism of cell death: apoptosis, autophagy and necrosis.

Cancer therapy is often targeted to induce apoptosis. Aggressive forms of cancer are less responsive towards cancer therapy because of their resistance to cell death pathways. Tumor cells also activate the expression of anti-apoptotic proteins like Bcl-x and Bcl-2 (Gutschner and Diederichs 2012).

The apoptotic cascade consists of the intrinsic and the extrinsic pathway. The intrinsic pathway processes intracellular signals, such as mitochondrial stress. The extrinsic pathway is caused by extracellular signals, such as the Fas ligand binding to its receptor. These pathways are responsible for activating caspases (caspases 8 and 9) which initiate a series of proteolytic events and thus activate the process of apoptosis. The intrinsic process of apoptosis is usually co-ordinated by balancing pro- and anti-apoptotic members of the Bcl family of proteins, such as Bcl-2 (Mcl-1, Bcl-w). They inhibit apoptosis by inactivation of pro-apoptotic members such as Bak

and Bax which cause disruption in the outer mitochondrial membrane (Gutschner and Diederichs 2012).

The second mechanism of cell death is autophagy, which is activated as a response to different cellular stresses, such as low levels of nutrients. The mTOR signaling pathway, which is down regulated during starvation, is an inhibitor of autophagy. The process usually generates energy in order to carry out cellular biosynthesis processes by breaking down cell organelles. When autophagy persists, it can also cause cell death. The third mechanism involved in cell death is necrosis, which is usually referred to as uncontrolled cell death (Gutschner and Diederichs 2012).

1.1.6 Mutational analysis of human breast cancers: Role of tumour suppressor and oncogenes

Breast cancer usually results from a series of genetic alterations, which cause the activation of oncogenes and inactivation of tumor suppressor genes. These genetic alterations result in genomic instability, increase in cell proliferation, hindrance in differentiation and a lack of apoptosis. Formation of tumours involves translocation of chromosomes, mutations, substitutions or deletions. Compared to normal growing cells, cancerous cells lack the ability to sustain the wholeness of the genome. (Felsher 2004).

Tumour suppressor genes serve as gatekeeper genes that regulate proliferation under normal conditions. Upon DNA damage these genes signal the cells to stop dividing further by promoting apoptosis or cell cycle arrest. Mutational inactivation of tumour suppressor genes leads to uncontrolled growth of cells. P53, PTEN and Rb (Retinoblastoma) are some examples of tumour suppressor genes. Oncogenes play important role in cancer progression and have been found to be mutated or overexpressed in many tumours. Activation of oncogenes results in rapid cell proliferation and causes disruption in the process of apoptosis and cellular differentiation, while their inactivation reduces cell proliferation or induced cell cycle arrest. RAS, ERK and MYC are some examples of oncogenes (Felsher 2004).

Breast cancer is usually caused by the accumulation of mutations in genes involved in cell growth or cell cycle. Most of these mutations are somatic while some are

inherited. These mutations include activating mutations in proto-oncogenes, which are dominant, while most inactivating mutations in tumor suppressor genes are recessive. Cells involved in breast cancer progression posses a unique ability to proliferate, metastasize and survive due to genomic instability which leads to further mutations and rearrangements of chromosomes (Eroles et al. 2012).

Oncogenes play a crucial role in growth factor signalling, including the signal transduction machinery (PIK3CA, AKT, Ras, Raf, Rheb), Tyrosine kinase receptors (EGFR/HER2), adaptors proteins (GAB2), intracellular proteins (SRC) and transcription factors (MYC, JUN and FOS). The functional loss of tumour suppressor genes involved in growth factor signalling, such as TSC1/2, PTEN and LKB1, increases cancer progression (Felsher 2004, Ali and Sjoblom 2009, Vogelstein et al. 2013).

Mutational analysis of breast cancer reveals several gatekeeping mutations that provide selective growth to normal cells. The further expansion in cell number as a consequence of the second mutation leads to the spread of tumour to distant organs (Previati et al. 2013). These types of mutations are termed as driver mutations. The genes which contain driver mutations in breast cancer are TP53, PTEN, PIK3CA, CDKN1B and ERBB3 (Liang et al. 2006). In general at chromosomal and nucleotide level healthy cells also posses different types of genetic alterations upon division, but they are designed to undergo apoptosis when mutations become deleterious (Vogelstein et al. 2013). On average one third of cancer genes are translocated, mutated or their expression is deregulated. Somatic mutations frequently occur in the majority of genes involved in the breast cancer pathway. In breast tumours about 33 to 66 genes show somatic mutations, among them 95% are single base substitution, while the rest of them are either deletions or insertions (Ali and Sjoblom 2009).

1.1.6.1 Breast cancer mutations

Among the diagnosed cases of Breast cancer 93-95% are sporadic while 5-7% are familial breast cancer. In familial breast cancer, 25% of cases exhibit germ line mutations in BRCA1 and BRCA2 while 5% contain mutations in other high susceptibility genes such as Tp53, PTEN and CDH1. Somatic mutations in BRCA1

(61%) are mainly found to be associated with Basal-like sporadic breast cancer (Anders and Carey 2009).

Other genes with somatic mutations found in breast cancer are GATA3 (trans-acting T-cell-specific transcription factor GATA-3 or GATA-binding factor 3) and TP53. Other mutations found in breast cancer are: CDH1 (cadherin-1), RB1 (retinoblastoma 1), MAP3K1 (mitogen-activated protein kinase kinase kinase 1 or MAPK/ERK kinase kinase 1), SMARCD1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1), NCOR1 (nuclear receptor corepressor 1), CASP8 (caspase 8, apoptosis-related cysteine protease), MAP3K13 (mitogen-activated protein kinase kinase kinase 13), CDKN1B (cyclin-dependent kinase inhibitor 1B (p27, Kip1), FOXA1 (forkhead box protein A1), TBX3 (T-box transcription factor), SF3B1 (splicing factor 3b, subunit 1), MYH9 (myosin heavy chain 9), AGTR2 (angiotensin II receptor, type 2), LDLRAP1 (low density lipoprotein receptor adaptor protein 1), STMN2 (stathmin-2), CBFB (core-binding factor subunit beta) with its associated partner RUNX1 (runt-related transcription factor 1), PTEN (phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatise) (Previati et al. 2013, Cornejo et al. 2014).

1.2 Signal transduction by the insulin and growth factor pathways in breast cancer

1.2.1 Role of tyrosine kinase receptors

Tyrosine kinase receptors play a crucial role in transmitting growth signals to the cell and serve as a major determinant of cell destiny (Sasaki et al. 2013). Tyrosine kinase receptors are involved in regulation of various cellular functions such as DNA replication, cell proliferation, transcription, DNA damage repair, metastasis of cancer, apoptosis, cell cycle advancement, development and differentiation (Roskoski 2014). Overexpressed and genetically altered forms of tyrosine kinases can cause oncogenic transformation in the cell. These mutations are usually found in the intracellular domain of growth factor receptors (Masuda et al. 2012). Therefore tyrosine kinase inhibitors (TKIs) which usually interact with the ATP

binding site of the tyrosine kinase domain are regarded as a promising class of cancer drugs (Ali and Sjoblom 2009).

The pioneering work done by Stanley Cohen in the growth factor field, led to the discovery of EGF (epidermal growth factor) and EGFR (epidermal growth factor receptor). This resulted in further identification of 14 families of tyrosine kinases receptors such as IGF-1R (Mendelsohn and Baselga 2000, Masuda et al. 2012). The IGF-1R and insulin receptor are activated by IGF-1, IGF-2 and insulin respectively and play a crucial role in stimulating signaling pathways involved in cell proliferation and survival (Werner and Bruchim 2012). EGFR was first identified as a transmembrane tyrosine kinase receptor and serves as a potent element in epithelial malignancies. It is by far the best examined signalling family in oncology (Earp et al. 2003).

1.2.1.1 EGFR signaling in breast cancer

Signalling pathways serve a crucial role in the regulation of cause, existence and spread of tumour growth. These pathways mediate biological signals to the cell and regulate various functions (Santen et al. 2002). Among other malignancies, breast cancer is one of the most complex diseases, with several stages of altered signalling pathways (Hadad et al. 2008). Under normal circumstances the level of EGFR is strictly regulated according to cell demand in order to maintain the balance of cellular functions (Sasaki et al. 2013).

1.2.1.1.1 Types of EGFR

The gene family of EGFR also called HER1 (human EGFR receptor 1) and ErbB1 (erythroblastosis virus) consist of four members: EGFR (ErBb1,HER1), ErBb2 (HER2,nue), ErBb3 (HER3) and ErBb4 (HER4) (Roskoski 2014). EGFR receptors consist of intracellular or cytoplasmic domains, which exhibit tyrosine kinase activity. They also have a C terminal tail, which contains several residues of tyrosine that are involved in initiation of signaling pathways upon phosphorylation. The extracellular or ligand binding domain is connected to the transmembrane hydrophobic domain (Roskoski 2014). The cytoplasmic tyrosine kinase domains of EGFR receptors are very similar, except for HER3 which lacks kinase activity, while the extracellular

domains are least conserved and have variable ligand binding affinities (Normanno et al. 2006).

1.2.1.1.2 Types of EGFR ligands

The family of peptide growth factors that bind to EGFR falls into three groups. The first group includes EGF, amphiregulin (AR) and transforming growth factor α (TGF-α), the second group consists of heparin-binding growth factor (HB-EGF), betacellulin (BTC) and epiregulin (EPR) and third group contains neuregulins (NRG-1, NRG-2, NRG-3 and NRG-4) (Mendelsohn and Baselga 2000). None of these ligands binds to HER2, while seven ligands bind to HER1 and HER4 and only two bind to HER3 (Roskoski 2014).

Several possible combinations of interaction exist between the four receptors and their 10 ligands, which lead to a diversified role of EGFR in signaling. Activation of the same receptors by different ligands induces distinct signaling pathways (Mendelsohn and Baselga 2000, Earp et al. 2003). Types of ligands and receptor complexes greatly influence the resulting activated signaling pathways (Normanno et al. 2006).

1.2.1.1.3 Mode of receptor activation

Binding of a ligand to the extracellular domain of EGFR receptors stimulates receptor activation, which promotes the formation of homo and heterodimer complexes and leads to the activation of tyrosine kinase domain (Gala and Chandarlapaty 2014). This is true for all EGFR receptors except for HER2, which only becomes activated when in a complex with another EGFR. Receptor activation favours the conformational change in the receptor resulting in the phosphorylation of C terminal tyrosine residues (Mendelsohn and Baselga 2000). These phosphorylated tyrosine residues provide docking sites for the phosphotyrosine binding (PTB) domains and Src homology 2 (SH2) comprising proteins. Interaction of these proteins results in the initiation of transduction pathways (Normanno et al. 2006).

1.2.1.1.4 EGFR mutations in breast cancer

The initial link between cancer and increased expression of receptors was found to be in EGFR (Roskoski 2014). The increased expression of EGFR and EGF is associated with many malignancies and they also have the potential to cause oncogenic transformation. EGFR is known to be amplified at gene level, while mutations in tyrosine kinase domain of EGFR are found to be associated with carcinogenesis (Normanno et al. 2006). In cancer EGFR levels are upregulated due to an extended yield of ligand or might be due to mutation in EGFR which leads to level of continuous production (Sasaki et al. 2013). Around 50% of Basal like subtype cases have increased expression of EGFR (Earp et al. 2003, Masuda et al. 2012).

1.2.1.1.4.1 Role of HER2

HER2 is overexpressed and amplified at gene level in 20-25% of breast cancers, leading to poor prognosis. Therefore HER2 is considered as a well recognized therapeutic target for breast cancer treatment (Earp et al. 2003). HER2 activating mutations are found in around 1.6% of patients (Roskoski 2014).

There is no known suitable ligand for HER2, therefore it forms heterodimers with other receptors. HER2 is the most active as it occurs in dimer-ready form and heterodimerized with other receptors (Earp et al. 2003). HER3 which lacks kinase activity prefers HER2 for dimerization, forming a HER2/HER3 complex. This dimer receptor complex is one of the strongest in stimulating the signaling cascades of PI3K/AKT and Ras/Raf/MAPK pathways (Normanno et al. 2006). HER3 induces kinase activity of HER2 by stimulating autophosphorylation of six tyrosine residues of C terminus of HER3.The terminus then interacts with p85 subunit of PI3K leading to activation of the PI3K signaling cascade, which is involved in carcinogenesis. Thus targeting PI3K pathway receptor tyrosine kinases may provide an effective tool in breast cancer treatment (Gala and Chandarlapaty 2014).

The EGFR inhibitors which act primarily by blocking ligand interaction are used as the best directed agent against breast cancer treatment. The combined targeting with EGFR inhibitors and anti-HER2 (MAbs), provide a more novel approach for the treatment of breast cancer (Normanno et al. 2006, Masuda et al. 2012).

1.2.1.2 Downstream signaling of EGFR

The family members of EGFR propagate growth stimulating signals through the interaction of EGF (epidermal growth factor) ligand into the cell, stimulating the tyrosine kinase function of the receptor and leading to activation of the signaling cascade. Thus it leads to the initiation of several biochemical modifications which include cell proliferation, transcription and DNA synthesis. The downstream signaling pathway activated by EGFR involves the Ras/Raf/mitogen-activated protein kinase and the PI3K/mTOR/AKT pathway, the most common altered signaling cascade in breast cancer progression as shown in figure 1.1 (Sasaki et al. 2013).

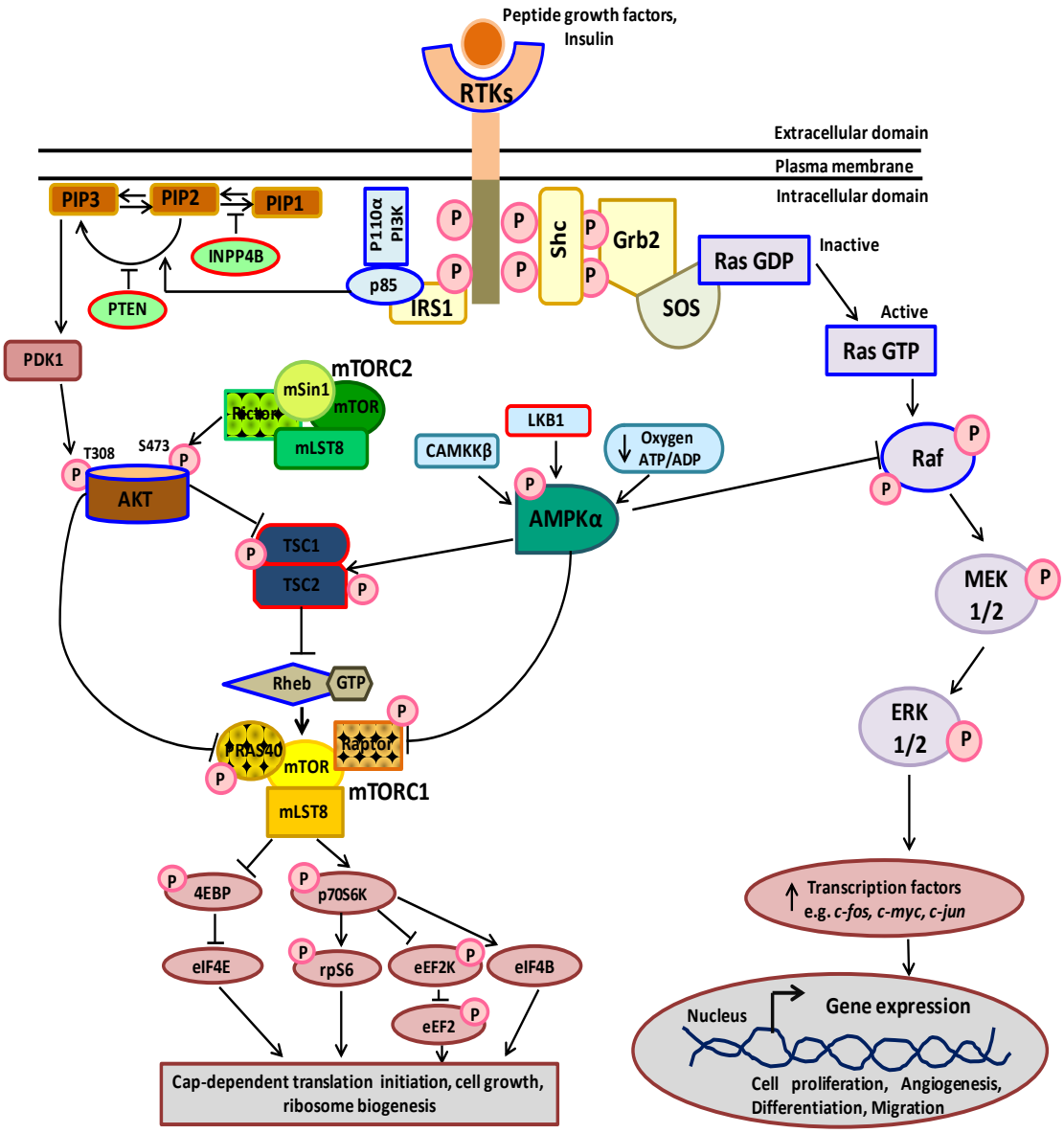


Figure 1.1: The signal transduction cascade downstream of receptor tyrosine kinase with AMPK as a negative regulator:

The growth factor/insulin stimulated RTKs activate the two most commonly altered signaling cascades of breast cancer. The PI3K/AKT and MAPK pathways are shown with their associated cellular proteins. Bars indicate inhibition while arrows represent activation. Both pathways are stimulated by RTKs through their respective adaptor proteins. The mTORC1 and mTORC2 a protein complexes of PI3K/AKT pathway mediates downstream signalling with the interaction of other protein partners. mTORC1 mediates translation regulation in response to amino acid, growth factors and cellular energy levels through phosphorylation of 4E-BP and S6K1. Upstream regulators of mTORC1 are AKT, AMPK and TSC-Rheb. AKT is activated through PI3K signalling, serving as a positive regulator of mTORC1 by inhibiting TSC complex and PRAS40 through phosphorylation while AMPK is activated by upstream kinases (LKB1, CAMKKβ) upon deprivation of cellular energy levels/oxygen. AMPK downregulates the activity of mTORC1 by stimulating the TSC complex and through Raptor phosphorylation. The TSC complex 1/2 act as a GTPase-activating protein for the Rheb, a small Ras-related GTPase involves in inhibiting mTORC1 activity by converting Rheb-GTP (active) form to Rheb-GDP (inactive) form. RTKs stimulation of of the MAPK pathway involvesthe conversion of inactive RAS into active RAS-GTP through SOS. Ras in GTP bound form activates Raf which further activates MEK1/2 by phosphorylation. Active MEK phosphorylates ERK1/2 which regulates various downstream cellular targets through the activation of various genes encoding transcription factors. AMPK inhibits MAPK signaling through BRAF phosphorylation. The potential tumour suppressors are highlighted with red borders while oncogenes are edged in dark blue.

Abbreviations: AMPK, adenosine monophosphate-dependent protein kinase; mTOR, mammalian target of rapamycin; PDK1, phosphoinositide-dependent protein kinase 1; PI3K, phosphoinositide 3-kinase; PRAS40, proline rich AKT substrate 40 kDa; or AKT1S1 (AKT substrate 1)); PIP3 (also known as PtdIns(3,4,5)P3), phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog; Raptor, regulatory associated protein of mTOR; Rheb, Ras homolog enriched in brain; Rictor, rapamycin-insensitive component of mTOR; S6K1, ribosomal S6 protein kinase 1; TSC1 and TSC2, tuberous sclerosis complex 1 and 2, ERK: extracellular signal-regulated kinase. MEK: mitogen-activated and extracellular signal regulated kinase kinase.

Receptor tyrosine kinases such as EGFR stimulate various growth related cellular functions through the interaction of peptide growth factors or EGFR associated ligands which leads to the activation of the signal transduction cascade (Normanno et al. 2006). In next section I will discuss the roles and links between these signaling pathways in breast cancer development, as well as the merit in targeting RTKs and signaling components of PI3K/AKT and Ras/MAPK pathways in the treatment of breast cancer.

1.2.1.2.1 The PI3K/AKT/mTOR pathway

1.2.1.2.1.1 What is mTOR

The large protein kinase complex, mammalian (or mechanistic) target of rapamycin (mTOR) is an extremely conserved serine-threonine kinase that plays a crucial role in many key cellular processes. These include cell proliferation, metabolism, differentiation, survival, growth, senescence and progression of metastasis by integrating extracellular and intracellular signals and are found to be deregulated in several cancers, such as breast cancer (Efeyan et al. 2012, Khan et al. 2013). mTOR signaling is inhibited by rapamycin, a macrolide ester, which interacts with intracellular cytoplasmic protein receptor FKBP12 (FK506 binding protein 12KDa). Upon entering into the cell, rapamycin forms a rapamycin/FKBP12 complex, which is associated with mTOR through FRB (FKBP12 rapamycin binding domain) (Laplante and Sabatini 2009). The PI3K/AKT/mTOR pathway, depicted in Fig 1.1, is activated by several upstream growth factor tyrosine kinase receptors such as EGFR (HER1), IGF-1R, insulin receptor (InsR) and HER2. In addition, it can also be activated through non-genomic estrogen signaling (Lauring et al. 2013, Martin et al. 2013). Insulin receptor substrate (IRS1-4) serves as an adaptor protein in insulin mediated activation of the PI3K signaling cascade (Jalving et al. 2010).

1.2.1.2.1.2 Multi-protein complexes of mTOR

mTOR is a kinase that regulates a complex signaling network and functions as a multidomain protein in complexes with several other proteins. It regulates ribosome biogenesis, protein synthesis and metabolism (Averous and Proud 2006, Guertin and Sabatini 2007). mTOR is found in two different multiprotein complexes known as mTORC1 (mTOR complex1) and mTORC2 (mTOR complex2) (Hadad et al. 2008). mTORC1 consists of mTOR, PRAS40 and Raptor while mTORC2 contains mTOR, mSIN1, Rictor and Protor1. mLST8 and Deptor are common in both complexes. mTOR kinase serves as the catalytic subunit in both protein complexes (Averous and Proud 2006, Laplante and Sabatini 2009).

1.2.1.2.1.2.1 mTORC2

The mTOR kinase in the mTORC2 is growth factor sensitive, but not sensitive to nutrients (amino acids) and rapamycin. Rictor serves as a scaffolding protein, which stimulates assembly and substrate binding to mTORC2 and interacts with Protor-1 (Wu et al. 2005, Laplante and Sabatini 2012). Deptor serves as a negative regulator of mTORC2 activity while mLST8 knockout is lethal and plays a crucial role in mTORC2 activity (Yonezawa 2004). mTORC2 phosphorylates and activates the AKT kinases.

1.2.1.2.1.2.2 mTORC1

mTORC1 is activated in response to amino acids, growth factors and cellular energy levels and is involved in the regulation of protein synthesis, ribosome biogenesis and promotion of cell growth (Dazert and Hall 2011, Laplante and Sabatini 2012). The mTOR kinase in mTORC1 is sensitive to the immune suppressive drug rapamycin. The rapamycin sensitivity of the complex is dependent on its interaction with the scaffolding protein Raptor, which facilitates the recruitment of mTORC1 substrates through their TOS motif to mTOR. In absence of Raptor, or after mutation of the TOS motif, mTOR kinase is unable to phosphorylate its downstream translational targets such as 4EBP and p70S6K (Yonezawa 2004, Yang and Guan 2007, Laplante and Sabatini 2009).

The mLST8 is not crucial for mTORC1 activity but enhances the interaction of mTOR and Raptor through the catalytic domain of mTOR. PRAS40 and Deptor negatively regulate mTORC1 activity. PRAS40 interacts with raptor through its TOS motif and competes for the binding site with other mTORC1 substrates, repressing the kinase activity of mTORC1. Phosphorylation of PRAS40 by mTORC1 on Ser183 demolishes the activity of PRAS40, which leads to its dissociation with mTORC1, thus enhancing the activity of mTORC1 (Yang and Guan 2007, Laplante and Sabatini 2009).

1.2.1.2.1.3 Phosphatidylinositol 2-kinases

mTOR belongs to a large family of proteins called PIKK (phosphoinositide kinase related kinases) and shares sequence similarity with the PI3K (phosphatidylinositol 3-kinase) lipid kinase family, which respond to growth factors and regulate mTOR

activity (Chiang and Abraham 2007, Hadad et al. 2008). The family of PI3K consists of three classes among which PI3K class 1A isoforms are mostly found to be involved in carcinogenesis. PI3K, a heterodimer enzyme, consists of a p110 catalytic and p85 regulatory subunits which exist in p110α, p110β, p110δ and p50α, p55α, p55γ, p85α and p85β isoforms respectively. Isoforms p110α, p110β and p110δ are encoded by PI3KCA, PI3KCB and PI3KCD and are most widely expressed (Miller et al. 2011).

Growth factors activate PI3K (phosphatidyl inositol-3-kinase) to mediate the phosphorylation of lipid messenger PIP2 (phosphatidylinositol-4,5-bisphosphate or PtdIns(3,4)P2) to PIP3 (phosphatidyl inositol-3,4,5-triphosphate or PtdIns(3,4,5)P3) (Yang and Guan 2007, Lauring et al. 2013). The function of PI3K is inhibited by PTEN (phosphatase and tensin homolog) and INPP4B (Inositol polyphosphate-4-phosphatase type II) by dephosphorylating PIP3 and PIP2 (Ciruelos Gil 2014). PIP3 acts as a second messenger and initiates downstream signaling through recruitment of PDK1 (PI3K dependent serine/threonine kinases) and AKT at the cell membrane. This is achieved through an interaction with PH (pleckstrin homology) domain of PDK1, which partially activates AKT through phosphorylation at T308 (threonine 308). The full activation of AKT is mediated by mTORC2 by phosphorylation on Ser473, which might be recruited through mSINI (Yang and Guan 2007, Yap et al. 2008). AKT (PKB or protein kinase B) is a serine threonine kinase involved in the regulation of cell growth, proliferation and apoptosis (Laplante and Sabatini 2009).

1.2.1.2.1.4 Regulation of mTORC1 by upstream kinases

mTOR serves as a main controller of growth and proliferation, which conveys signals to its downstream targets in response to growth factors and nutrient availability. Growth factor mediated inhibition of TSC1/2, which in turn activates Rheb-GTP, leads to the initiation of mTORC1 activity (Gingras et al. 2001, Saini et al. 2013).

1.2.1.2.1.4.1 TSC mediated RHEB pathway

The Tuberous Sclerosis complex (TSC 1 and TSC 2) serves as a negative regulator of mTORC1 activity and plays a crucial role in the regulation of mTOR signaling

through AMPK and AKT (Yonezawa 2004). TSC is a heterodimer which consists of a complex of two interacting proteins called TSC1 (hamartin) and TSC2 (tuberin) (Dazert and Hall 2011). TSC1/2 is a guanosine triphosphatase or GTPase activating protein (GAP) for a small GTPase, Rheb (Ras homolog enriched in brain). Rheb activates mTORC1 through its active GTP bound state (Laplante and Sabatini 2009). TSC1/2 complex converts active GTP bound Rheb into its inactive GDP bound form and thus negatively regulates mTORC1 (Inoki et al. 2012, Laplante and Sabatini 2012).

1.2.1.2.1.4.2 AKT: A positive regulator of mTORC1 activity

AKT is involved in cell survival, inhibition of apoptosis, cell growth and proliferation by positively regulating the mTORC1 kinase activity (Gingras et al. 2001, Laplante and Sabatini 2009). mTORC2 activates AKT signaling by PI3K in response to growth factors. AKT upstream of mTORC1 is involved in translational regulation of its downstream signaling targets in response to nutrients (Guertin and Sabatini 2007, Yang and Guan 2007). AKT promotes mTORC1 kinase activity by two independent pathways: the TSC-Rheb pathway and the PRAS40-mTORC1 pathway.

AKT inhibits the activity of the TSC complex through phosphorylation of TSC2 on multiple sites leading to upregulation of mTORC1 kinase activity, linking PI3K-AKT to TSC-mTORC1 signaling pathways (Laplante and Sabatini 2013). Overexpression of AKT leads to hyperphosphorylation of translational targets such as 4EBP and p70S6K, through inactivation of TSC1 and TSC2 heterodimer (Yonezawa 2004).

AKT also positively regulates mTORC1 kinase activity, independent of the TSC-Rheb pathway, through directly phosphorylating the PRAS40 (proline-rich Akt substrate 40 kDa or AKT1S1 (AKT substrate 1)) on T246 near its carboxyl terminal region, which decreases its interaction with mTORC1 kinase (Yang and Guan 2007). PRAS40 suppresses RHEB-GTP activation of mTORC1 and remains associated with mTORC1 (Guertin and Sabatini 2007). Thus AKT promotes mTORC1 kinase activity by stimulating the activity of RHEB-GTP and releasing the inhibitory effect of PRAS40 from mTORC1 by phosphorylation (Xu et al. 2012). PRAS40 suppresses the phosphorylation of mTORC1 substrates such as 4EBP and S6K1 by competing for the

same kinase raptor-mTOR among these substrates. Therefore hyperphosphorylation of PRAS40 reduces 4EBP and S6K1 phosphorylation and vice versa (Yang and Guan 2007).

1.2.1.2.1.4.3 AMPK: A negative regulator of mTORC1 activity

mTOR is a downstream target of AMPK (5'-adenosine monophosphate-activated protein kinase) which regulates cell metabolism, cell growth and proliferation in response to signals from the environment such as oxygen, nutrients and energy status (Xu et al. 2012) (Inoki et al. 2002, Laplante and Sabatini 2009). The process of protein synthesis is highly energy consuming and has to be increased during cell growth in response to growth factor signals. However if there are insuffient nutrients or inadequate amount of energy, the rate of protein synthesis needs to be reduced and cell growth prevented (Xu et al. 2012). AMPK is regulated in response to changes in the intracellular ratio of AMP/ATP or due to elevated intracellular levels of Ca2+ (Kim and He 2013). Favorable conditions stimulate cell growth through mTOR activation by inhibiting AMPK (Inoki et al. 2012). AMPK consists of an α catalytic subunit, while β and γ are regulatory subunits. Upstream kinases such as LKB1 (serine/threonine kinase 11), a tumour suppressor, and CaMKKβ (Calcium/calmodulin-dependent protein kinase kinase-b) family kinases play an essential role in AMPK activation, by phosphorylating the catalytic α subunit at threonine residue 172 (Thr-172). In mammals, LKB1 is the critical enzyme for AMPK activation and thus provides an initial link of AMPK to cancer (Hadad et al. 2008).

AMPK is involved in regulating the balance of intracellular energy levels through modulating the phosphorylation state of its substrate by catabolic or ATP generating processes such as fatty acid oxidation and glycolysis. AMPK also regulates energy levels through inhibiting anabolic processes to reduce the consumption of ATP, such as inhibiting protein and fatty acid synthesis (Shen et al. 2013). AMPK becomes activated during cellular stress conditions, including low oxygen or hypoxia, which lead to a low ratio of ATP/ADP (Laplante and Sabatini 2009). The rate of cell growth is therefore highly regulated by mTOR, in relation to

the availability of nutrients and energy in co-ordination with AMPK (Chiang and Abraham 2007).

AMPK negatively regulates mTOR by stimulating the activity of the TSC by phosphorylating TSC2 on S1345 and T1227 which inhibits the activity of Rheb by activating TSC GTPase activity and converting Rheb into its GDP bound state (inactive state) (Xu et al. 2012). AMPK also suppresses mTORC1 activity through Raptor phosphorylation which may destabilize the complex (Laplante and Sabatini 2009). Thus mTORC1 efficiently regulates protein synthesis by blocking the phosphorylation of 4EBP and S6K1 through AMPK mediated inhibition of mTORC1 under low energy or stress conditions, mediated by TSC-mTORC1 pathway (Yang and Guan 2007). AMPK also mediates the inhibition of mTORC1 in response to energy reduction through raptor phosphorylation, which disrupts its association with mTOR and thus inhibits substrate binding (Laplante and Sabatini 2009, Inoki et al. 2012).

1.2.1.2.1.5 Downstream signaling of mTOR

The best characterized function of downstream targets of the mTORC1 signaling pathway is the regulation of translation machinery in response to availability of nutrients, especially amino acids, through an unidentified mechanism. It acts independently of the TSC complex mediated pathway (Laplante and Sabatini 2009, Laplante and Sabatini 2012). mTOR not only changes the phosphorylation state of translation initiation factors, but also influences levels of components involved in the translation process by controlling their rate of synthesis (Gingras et al. 2001). The deregulation in FRAP/mTOR signaling pathway due to altered levels of signaling components, also leads to translational deregulation, linked with an increase in cell growth. Disrupted FRAP/mTOR signaling contributes to translational activation of mRNAs involved in cell cycle progression as well as inhibitors of apoptosis (Dowling et al. 2007). Deregulation of translational pathways is thought to lead to cell transformation, as a result of the altered production of proteins that are involved in promoting and suppressing cancer (Clemens 2004).

The downstream effectors of mTOR signaling which are directly or indirectly phosphorylated by mTOR are 4EBP1 (4E-binding protein), eIF4G and S6K kinases (p70 ribosomal protein S6 kinase1 and 2). S6K kinases then phosphorylate eIF4B, eEF2K (Eukaryotic elongation factor-2 kinase) and ribosomal protein 6, thus promoting cell growth by enhancing cap dependent translation, elongation and ribosome biogenesis (Sonenberg and Hinnebusch 2009, Laplante and Sabatini 2012).

eIF4G1 is phosphorylated at several sites by mTORC1, which stabilizes the complex of eIF4G1-eIF3 and leads to efficient recruitment of the 40S ribosomal subunit. However, the direct phopshorylation of eIF4G1 by mTOR is still uncertain (Averous and Proud 2006, Mamane et al. 2006, Thoreen 2013). mTORC1 phosphorylates and activates the p70 S6 kinases (S6K1 and S6K2). The most abundant substrate of S6K1 is RpS6 (ribosomal protein 6), a phosphoprotein (Hershey 2010, Komelkov 2012). S6K1 is also reported to be involved in promoting tumour growth and its expression is elevated in several cancers (Mamane et al. 2006). The phosphorylation of eIF4B by S6K1 not only enhances the helicase activity of eIF4A but also increases the association of eIF4B with eIF3, thus leading to efficient initiation of cap dependent translation by promoting the recruitment of the translation initiation complex (Gingras et al. 2001, Sonenberg and Hinnebusch 2009).

1.2.1.2.1.5.1 eIF4E/4E-BP

In a complex process of protein synthesis, eIF4E is a main regulator during the rate limiting step of translation initiation. eIF4E is also involved in tumorigenesis and found to be deregulated in a variety of cancers (Mamane et al. 2004, Averous and Proud 2006). eIF4E specifically binds to the 5’ cap structure of an mRNA as a component of the trimeric complex of eIF4F with eIF4G (a scaffolding protein) and eIF4A (an RNA helicase) (Montanaro and Pandolfi 2004). High levels of eIF4E enhance the translation of specific mRNAs involved in cell growth (c-myc), cell cycle progression (cyclin D1) and in angiogenesis (VEGF). Thus eIF4E is a potential proto-oncogene (Montanaro and Pandolfi 2004, Thoreen 2013).

The availability of functional eIF4E is regulated through the phosphorylation state of 4EBP (4E-binding proteins) also known as PHAS-I (phosphorylated heat and stable protein, insulin stimulated). 4EBP1 acts as a translation repressor in a hypophosphorylated state, by inhibiting the assembly of eIF4F complex by competing for the binding site with eIF4G on the dorsal face of eIF4E. In this way, it inhibits cap dependent translation. 4EBP in its hyperphosphorylated state dissociates from eIF4E, allowing eIF4G to effectively interact with eIF4E, which leads to translation initiation (Bjornsti and Houghton 2004, Hershey 2010). Consequently, mTOR mediates translation regulation by stimulating cap dependent translation through 4EBP1 phosphorylation in response to extracellular stimuli (Yang and Guan 2007). In its hypophosphorylated state, 4EBP acts as a tumor suppressor by reducing the levels of functional eIF4E. This leads to downregulation of specific mRNAs, which become translationally upregulated due to increased levels of eIF4E (Meric and Hunt 2002). Thus high levels of 4EBP revert the oncogenic phenotype of transformed cells caused by elevated levels of eIF4E (Hershey 2010).

1.2.1.2.2 Raf/MEK/Erk1/2 pathway (MAPK)

1.2.1.2.2.1 Role of MAPK (ERK1/2)

The MAPK (mitogen-activated protein kinases) is a family of serine threonine protein kinases, also known as the ERK pathway. This is an extremely conserved signal transduction pathway among eukaryotes that incorporates various extracellular signals into the cellular machinery. ERK consist of two isoforms ERK 1 and 2, which have 85% similarity. However, ERK 2 is the one that is predominantly implicated in carcinogenesis (Santen et al. 2002, Deschenes-Simard et al. 2014). MAPK mediates signal transduction depending upon stimulus and cell type, regulating cellular processes ranging from cell proliferation and survival to activating tumor suppressor events, such as apoptosis and cell differentiation (Dhillon et al. 2007, Deschenes-Simard et al. 2014). Among the three MAPK pathways which are found in humans, the one which consists of MEK 1/2 (MAP/ERK kinase) and ERK1/2 (extracellular signal regulated kinases 1 and 2) is heavily implicated in breast cancer (Saini et al. 2013). ERK 1 and ERK 2 are localized in

various cellular places such as specific parts of plasma membrane, microtubule associated cytoskeleton and nucleus (Santen et al. 2002).

1.2.1.2.2.2 ERK signaling cascade

The ERK cascade consists of three sequential series of kinases: Raf1 (MAPKKK), MEK 1/2 (MAPKK) and ERK 1/2 (MAPK). Growth of estrogen independent breast cancer subtypes is dependent on peptide growth factors as major extracellular stimuli, which activate ERK signaling through tyrosine kinase receptors, stimulating phosphorylation of adaptor proteins such as Shc (Santen et al. 2002). The Shc/receptor complex interacts with Grb2 and SOS (son of sevenless). Grb2 acts as scaffold protein that connects RTKs to downstream components. Interaction of Grb2 or Shc adaptor proteins to RTKS results in stimulation of Raf/MEK/ERK signalling (Adams et al. 2012). SOS is a Ras-guanine nucleotide exchange factor that facilitates converstion of GDP-RAS to GTP-RAS. RAS is an oncoprotein that plays crucial roles in cellular proliferation and cancer progression and consists of three family members *KRas, NRas and HRas (Saini et al. 2013)*. The first kinase effector of GTP-RAS is Raf-1, which exists in three isoforms (ARaf, BRaf, CRaf or Raf-1). The GTP-RAS mediated phosphorylation activates Raf. Activated Raf phosphorylates a second downstream kinase MEK that also exists in two isoforms MEK 1 and 2. BRaf is most effective in activating MEK, followed by Raf-1 and ARaf. MEK activation leads to stimulation of ERK through phosphorylating tyrosine and threonine residues. Activated ERK 1 and 2 phosphorylate a large number of downstream targets that have been linked with gene regulation (Santen et al. 2002).

1.2.1.2.2.3 Downstream signaling events of ERKs

ERKs signaling involve the regulation of many downstream cellular events through phosphorylation of various key cytoplasm and nuclear players. Targets of ERKs signaling include cytoskeleton proteins, kinases, phosphatases and various transcription factors. The diverse biological roles of ERK signaling are dependent upon the signaling intensity and localization of ERK (Dhillon et al. 2007, Roskoski 2014).

ERKs signaling mediated activation of other protein kinases such as RSK via phosphorylation, leads to stimulation of various downstream cellular targets of ERKs cascade that are involved in transcriptional regulation (Santen et al. 2002). Nuclear translocation of ERKs upon activation plays a crucial role in stimulation of several transcription factor encoded genes, including *c-fos, c-myc, ATF-2* (activating transcription factor 2) and *c-jun,* which also promote cell proliferation. Activation of the ERK cascade also leads to phosphorylation of histone 3 and HMG-14. Thus ERKs signaling regulates gene expression and contributes to chromatin remodeling (Santen et al. 2002).

Sustained ERK signaling not only leads to stable activation and high expression of proliferation linked transcription factors, but it also stimulates CDKs (cyclin-dependent kinase) such as cyclin D1. This helps to promote cell cycle progression and also inhibits anti-proliferative gene expression (Fowler et al. 2011, Deschenes-Simard et al. 2014). However, a hyperactive ERK cascade blocks cell cycle progression by activating CDKs inhibitors such as p27 and p21 (Dhillon et al. 2007).

1.2.1.2.2.4 AMPK: attenuates MAPK signaling

The molecular link between Ras-Raf-MEK-Erk and LKB1-AMPK pathways is complicated and still not entirely clear. These two pathways are a major link between the regulation of cell growth and proliferation, in response to cellular energy status or under metabolic stress (Zheng et al. 2009). BRAF is found to be mutated in several malignancies. The amino acid change in the kinase domain of BRAF leads to mutant V600E involved in continuous signaling and activation downstream of MAPK pathway. The KSR (kinase suppressor of Ras), a scaffold protein of MAPK pathway, stimulates RAF mediated MEK phosphorylation. The LKB1 mediated activation of AMPK by AICAR leads to phosphorylation of BRAF at Ser729, which interrupts the interaction of BRAF-KSR and CRAF, resulting in inhibition of MAPK signaling pathway (Shen et al. 2013). The relationship between LKB1-AMPK and MAPK signaling pathway is attenuated in cancerous cells harboring various genetic aberrations. Conversely, the MAPK signaling cascade reduces AMPK signaling through phosphorylation mediated inactivation of the upstream Ser/Thr kinase LKB1 at Ser428 by ERK and p90RSK in BRAF mutant V600E cells, providing

resistance to metabolic stress. However in cells with wild type BRAF, AICAR induces AMPK activation as normal (Esteve-Puig et al. 2009). Phosphorylation of BRAF by AMPK also leads to inhibition of cell proliferation by stimulating cell cycle arrest, due to elevated levels of cell cycle inhibitors such as p21 (Petti et al. 2012).

1.2.1.2.3 Genetic alterations in downstream signaling pathways

Growth factor mediated signalling pathways such as PI3K/AKT/mTOR and Raf/MEK/ERK are important in cancer progression, as many key players of signalling pathways are found to be deregulated or expressed constitutively in breast cancer (Hadad et al. 2008, Saini et al. 2013). Alterations in signals regulated by membrane receptors or TRKs are often due to changes in the amount of receptors found on the cell surface or to modifications that lead to continuous activation of signalling pathways, without the presence of the ligand. Pathways like PI3K/AKT, AMPK, mTOR, IGFIR, estrogen receptors and HER2 are under intense investigation as therapeutic targets in breast cancer treatment (Eroles et al. 2012, Werner and Bruchim 2012).

1.2.1.2.3.1 Mutation analysis of mTOR

The PI3K/mTOR pathway is found to be deregulated in 70% of breast cancer (Miller et al. 2011, Lauring et al. 2013). Constitutive signaling of this pathway is either due to overexpression of RTKs (HER2, IGF-IR), or somatic alterations in PIK3CA (p110α) and its downstream effectors like AKT, TSC1/2 (Calkhoven et al. 2002, Khan et al. 2013). Activation of the mTOR pathway also leads to hyperphosphorylation of downstream translational targets such as S6K and 4EBP1, resulting in hyper-activated translation and growth and proliferation of tumor cells (Gingras et al. 2001, Martin et al. 2013). The catalytic subunit of PI3K kinase, PI3KCA is found to be mutated in 36% of breast cancer, with 80% genetic alteration in the kinase domain of the p110α subunit and frequent combination with HER2 overexpression or PTEN loss (Ali and Sjoblom 2009, Martin et al. 2013, Saini et al. 2013). The frequency of genetic alterations of PI3K signaling pathway in different subtypes of breast cancer is summarized in table 2.

Table 2: Genetic alterations of the PI3K/AKT/mTOR pathway in breast cancer subtypes

Type of alteration	Gene (Protein)	Consequence on signaling	Frequency in breast cancer subtypes (%)		
			ER+ (Luminal)	Basal (TN)	HER2+
Amplification or activating mutations	*ErbB2* (HER2)	Hyperactivation of HER2 signaling (PI3K, MEK)	10	0	100%
	EGFR	Hyperactivation of EGFR signaling (PI3K, MEK)	-	-	0.8
	AKT1	Hyperactivation of AKT	2.6-3.8	0	0
	AKT2	Hyperactivation of AKT	2.8 in all subtypes		
	PDK1	Hyperactivation of PDK1 (AKT,mTORC1)	22	38	22
	PIK3CA (P110α PI3K)	Hyperactivation of PI3K signaling	28-47	8-25	23-33
	IGFIR & INSR (IGF-IR, InsR)	Activates IGF-IR/InsR signaling	41-48	42	18-64
	RPS6K1 (p70S6K)	unknown	3.8-12.5 in all subtypes		
Loss of function mutations	*PTEN*	Hyperactivation of PI3K signaling	29-44	67	22
	INPP4B	Hyperactivation of PI3K signaling	10-33	53	54

Abbreviations: AKT, protein kinase B; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; ER,estrogen receptor; IGF-1R, insulin-like growth factor-1 receptor; INPP4B, inositol polyphosphate-4-phosphatase, type II; InsR, insulin receptor; MEK, mitogen-activated
protein kinase kinase; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase

Adapted from: (Miller et al. 2011, Saini et al. 2013, Ciruelos Gil 2014, Cornejo et al. 2014).

1.2.1.2.3.2 Mutational analysis of MAPK pathway

The MAPK pathway is often over expressed in breast cancer but rarely found to be mutated in breast cancer progression (Santen et al. 2002). The basal like subtype of breast cancer contains the largest increase in expression of ERK signaling molecules (Saini et al. 2013). RAS is the most general human oncogene found to be mutated in 30% of cancers, while 4-6% of breast cancers contain an activating mutation in KRAS, which leads to hyperactivation of ERK signaling (Miller et al. 2011, Deschenes-Simard et al. 2014). BRAF is found to be mutated in 3% of breast cancers (Saini et al. 2013). MEK mutation is even more uncommon, while ERK has never been reported

as being mutated. Overexpression or activating mutations in tyrosine kinase receptors and the continuous production of ligands with activated upstream oncogenes (RAS and RAF) leads to constitutive stimulation of ERK signaling and promotes breast tumour progression (Dhillon et al. 2007, Saini et al. 2013, Deschenes-Simard et al. 2014).

1.2.1.2.4 Interference between PI3K and ERK pathway

The cross-talk between the PI3K/mTOR and MAPK pathways is complicated and not entirely clear. The PI3K/mTOR pathway is mainly activated through the interaction of P85, a regulatory subunit of PI3K with HER2 and EGFR. The Ras/Raf/mitogen-activated protein kinase (MAPK) pathway is mainly induced by Shc or Grb2 adaptor proteins (Normanno et al. 2006). The kinases of these two pathways highly influence the functional activity of each other through modulating the phosphorylation state. Ras is a key activating molecule of MAPK signaling pathway and regulates PI3K activity through various mechanisms. Ras inhibits the activity of PTEN, a tumour suppressor gene, thus stimulating PI3K (Saini et al. 2013). Raf (B-Raf and Raf-1) the downstream component of MAPK signaling is inhibited by AKT and Rheb, which attenuates MAPK signaling. In addition, ERK1/2 mediates the inhibition of TSC complex, a negative regulator of mTOR activity (Saini et al. 2013). Consequently, in the presence of growth factors, the inhibition of one pathway ultimately stimulates the other pathway due to failure of the feedback mechanisms. For instance, it has been reported that inhibiting AKT without blocking mTORC1, stimulates signaling from RTKs which leads to active PI3K pathway through the S6-PI3K feedback pathway (Khan et al. 2013).

1.2.1.2.5 Therapeutic strategies of breast cancer

Current therapeutic strategies in breast cancer treatment are focused on targetting receptor mediated signaling such as hormone receptors or HER2 (Martin et al. 2013). Therefore TKI (tyrosine kinase inhibitors) for EGFR and HER2 such as Lapatinib are considered as a potential agent in HER2+ breast cancer (Martin et al. 2013, Vogelstein et al. 2013). Breast cancers which contain genetic alterations in signaling pathways such as mTOR/AKT and MAPK pathway have resistance for

anti-HER2+ and endocrine therapy, therefore targeting either signaling pathway in combination with TKIs is considered as a standard therapy in breast cancer treatment (Ciruelos Gil 2014).

1.2.1.2.5.1 PI3K/mTOR/AKT inhibitors

Several mTOR/AKT pathway inhibitors are considered as potential therapeutic agents, which could be used in combination with traditional breast cancer therapy such as anti-HER2+, in addition to TKIs (Miller et al. 2011). In ER+ breast tumours, elevated levels of PI3K signaling lead to reduced expression of ER resulting in resistance to endocrine therapy. Therefore mTOR inhibitors could restore the sensitivity to endocrine therapy (Ciruelos Gil 2014). Five main inhibitors targeting PI3K pathway at various levels are summarized below:

1.2.1.2.5.1.1 PI3K inhibitors

PI3K inhibitors are classified into Pan-PI3K inhibitors and PI3K-isoform inhibitors. These inhibitors interact specifically with the ATP pocket and specifically act on class1A PI3K members. PI3K-isoform inhibitors act on different isoforms of the catalytic subunits (p110α, β, γ, δ) (Khan et al. 2013).

1.2.1.2.5.1.2 AKT inhibitors

This class includes ATP-competitive and allosteric AKT inhibitors. ATP-competitive inhibitors act towards three isoforms of AKT through competing with the ATP binding pocket. Allosteric AKT inhibitors interact with PH domain of AKT to induce a conformational change, thus preventing AKT translocation to the plasma membrane and inhibiting its activation (Miller et al. 2011).

1.2.1.2.5.1.3 mTOR inhibitors

1.2.1.2.5.1.3.1 Rapamycin

Rapamycin and its analogues (such as everolimus) form complexes with FKBP12 (FK506 binding protein 12KDa) through FRB (FKBP12 rapamycin binding domain), thus inhibiting mTORC1 signaling (Martin et al. 2013). However, there are

difficulties with the formulation of rapamycin and its analogs. It is therefore not considered a suitable agent for breast cancer treatment (Miller et al. 2011).

1.2.1.2.5.1.3.2 mTORC1/2 inhibitors

mTOR kinase inhibitors that bind to the ATP interacting site of the mTOR kinase domain target both mTORC1 and 2. RpS6, a downstream product of mTORC1, negatively feeds back to IRS1, which in turn diminishes the activity of PI3K. Such dual inhibitors of mTOR lead to complete inhibition of PI3K signaling (Lauring et al. 2013).

1.2.1.2.5.1.3.3 PI3K/mTOR dual inhibitors

Blocking mTORC1 signaling eases the negative feedback on PI3K activators. Therefore targeting PI3K is a more effective way of blocking PI3K/AKT/mTOR signaling. Increased expression of RTKs also stimulates other oncogenic signaling cascades such as MAPK pathway. Due to the similarity in kinase domains of PI3K and mTOR these agents can act as dual inhibitors. Thus combination treatments of TKIs with PI3K/AKT/mTOR inhibitors are expected to have increased effectiveness (Miller et al. 2011).

1.2.1.2.5.2 MAPK pathway inhibitors

Several drugs have been developed so far as targets of the MAPK pathway at various nodes. Currently in clinical trials are non competitive ATP MEK1/2 inhibitors (Selumetinib) and inhibitors of BRaf kinase (Sorafinib) (Saini et al. 2013, Deschenes-Simard et al. 2014).

The strategy of targeting the two most relevant deregulated cascades of breast cancer: PI3K/mTOR and MAPK pathway should provide a better clinical response in breast cancer treatment (Saini et al. 2013). Targeting PI3K pathway together with the MAPK pathway is more effective in restoring the sensitivity to ER+ breast cancer (Ciruelos Gil 2014).

1.2.1.2.5.3 Activating AMPK: a negative regulator of PI3K and MAPK pathway

Several activators of AMPK are currently considered as potential anti cancer drugs. Active AMPK is involved in the downregulation of crucial signaling pathways, which inhibits cell proliferation and survival. MAPK pathway inhibitors in combination with AMPK activators could potentially provide a better strategy for inhibiting cell proliferation in several cancers. Further investigation into evaluating the role of AMPK and MAPK pathways in carcinogenesis could provide better therapeutic options for breast cancer treatment (Zheng et al. 2009). Activators of AMPK such as metformin and its analogues AICAR and phenformin, which exerts their action through LKB1 kinase, are considered as major activators of AMPK (Hadad et al. 2008). AMPK activators stimulate apoptosis and reduce cell proliferation in basal-like and estrogen positive and HER2+ subtypes of breast cancer (Kim and He 2013).

1.3 Eukaryotic polyadenylation

In this thesis, I examine the potential of inhibiting polyadenylation as a mechanism of action for novel breast cancer drugs. The process of polyadenylation in mammalian mRNA was reported in 1970 (Penman et al. 1970, Rose and Jacob 1978). In mRNA metabolism the poly (A) tail plays an important role (Tian and Graber 2012, Goss and Kleiman 2013). As soon as the process of transcription starts, the pre-mRNA is processed in several steps. These include capping of an mRNA followed by splicing. The termination of transcription is coupled to the cleavage of the mRNA and the 3' end of the cleavage product is then polyadenylated (Kuehner et al. 2011, Sagawa et al. 2011).

Almost all of the eukaryotic pre-mRNAs undergo the process of cleavage and polyadenylation except histone mRNAs, which are only cleaved (Tian and Manley 2013). The poly (A) tail, a stretch of adenosine monophosphate residues at the end of an mRNA, stimulates translation, stability and localisation of mRNAs (Cappell et al. 2010, Cevher et al. 2010, Ozsolak et al. 2010). The addition of a poly A tail is considered a default process on the way to mRNA maturation, which is essentially the same for all non-histone mRNAs (Mirkin et al. 2008, Sagawa et al. 2011). Addition of a poly(A) tail to an mRNA is a two step process: it is first initiated with

an endonucleolytic cleavage followed by the addition of a poly(A) tail (Mirkin et al. 2008).

Cleavage and polyadenylation plays a vital regulatory role in mammalian gene expression. Besides being the mechanism of 3' end formation of mRNA, it is also required for the termination of transcription (Guhaniyogi and Brewer 2001, Nunes et al. 2010), in export of the mRNA from the nucleus (Huang and Carmichael 1996), in translation of the mRNA (Sachs et al. 1997), in removal of the terminal intron (Nunes et al. 2010) and in controlling the stability of the mRNA (Guhaniyogi and Brewer 2001, Eckmann et al. 2011).

1.3.1 Mechanism of nuclear polyadenylation

The initial polyadenylation of an mRNA takes place in the nucleus and is coupled with cleavage (Ghoshal and Jacob 1991). There are different *cis* and *trans* regulatory elements involved in the process of cleavage and polyadenylation, which are tightly coupled with transcription and translation (Moore and Proudfoot 2009, Tian and Graber 2012). It is generally believed that all polyadenylated mRNAs are appended with a tail of 230 adenosine residues at the cleavage site (Penman et al. 1970, Darnell et al. 1971).

1.3.1.1 Cis acting Sequence elements

The process of cleavage and polyadenylation is a well co- ordinated stage in 3'end processing, involving cis acting elements which are present both upstream and downstream of poly (A) signal. The four sequence elements in 3' end processing are the upstream element (USE, containing U(G/A)UA), the poly(A) signal, the GU or U rich downstream sequence (DSE) and the G rich auxilliary sequence (Zhao et al. 1999, Charlesworth et al. 2013).

The core polyadenylation signal (AAUAAA or AUUAAA) is usually present <30 nucleotides downstream of USE (upstream sequence element) which is identified by CPSF (cleavage and polyadenylation specificity factor) as shown in figure 1.3 (Ryan et al. 2004, Danckwardt et al. 2007). The canonical hexamer motif (AAUAAA or AUUAAA) in humans is 70-80% conserved. However in some cases, the process of cleavage and polyadenylation is mediated by a non-canonical poly (A) signal, as 20-

30% of mammalian genes do not have a conserved canonical poly (A) signal. The processes of alternative cleavage and polyadenylation are found to be more common in mRNAs containing a non-canonical poly (A) signal (Nunes et al. 2010). About 16% have AUUAAA while 20% contain single base variants A(A/U)UAAA and 10% of poly (A) signals lack relevant AAUAAA type sequence (Tian and Manley 2013). The downstream canonical sequence element (DSE) is G/U rich and is present <30 nucleotides downstream from the site of cleavage. It is bound by the 64-kDa subunit of the CstF (cleavage stimulation factor) through its RNA binding domain found in the N terminus (Colgan and Manley 1997, Zhao et al. 1999). The USE enhances the efficiency of cleavage and polyadenylation but it is not present in all mRNAs in which cleavage occurs such as in histone pre-mRNAs (Mandel et al. 2008). The USE stabilizes RNA interaction between CPSF and CstF, often determining the choice of cleavage site. The G-rich downstream auxillary sequence element which aids in cleavage site selection still lacks a binding partner (Charlesworth et al. 2013).

1.3.1.2 Trans acting protein factors

The process of nuclear polyadenylation is a multiple step event that involves several protein complexes of multiple subunits (de Klerk et al., 2012, Ghoshal and Jacob, 1991). Proteomics experiments indicate the cleavage and polyadenylation complexes consist of more than 80 proteins in humans (Shi et al. 2009, Nunes et al. 2010). The main components of these multi protein complexes are highly conserved and comprise several protein complexes flanking the cleavage site, including CPSF, CstF, cleavage factors I and II (CFI_m and $CFII_m$) and poly (A) polymerases (PAP), as summarised in table 3. For the process of cleavage and polyadenylation, the assembly of these protein complexes in mammals is initiated by the interaction of CPSF and CstF to core sequences on premature mRNAs (Zhao et al. 1999, Takagaki and Manley 2000). There are some scaffolding proteins that lack RNA binding function and only serve as a binding site for other regulatory elements such as symplekin and CstF-3 (Tian and Manley 2013).

The UGUAN motif found upstream of cleavage site at variable distance in 3'UTR recruits an additional heterodimeric core factor, $CFI_{m,}$ which stimulates the process of cleavage and polyadenylation in the absence of the conserved AAUAAA hexamer. The role of $CFII_m$ is still unknown (Danckwardt et al. 2008, Nunes et al. 2010).

Table 3: List of factors involved in mammalian 3' end cleavage and polyadenylation

Factors	Subunits (KDa)	HUGO Nomenclature	Features
CPSF (cleavage and polyadenylation specificity factor)	160	CPSF 1	Recognizes the poly (A) signal and interacts with CstF-77, PAP and CTD of RNAPII
	100	CPSF 2	Involved in 3' end mRNA processing
	73	CPSF 3	Endonuclease mediates the cleavage of pre-mRNA at 10-30 nucleotides downstream of the poly (A) signal
	30	CPSF 4	Binds to U rich elements on pre-mRNA
	Fip1L	-	In vitro, FipL1 is responsible for recruitment of PAP thus link CPSF1 and PAP
CstF (cleavage stimulating factor)	50	CstF 1	Mediates 3' end processing interacts with CTD of RNAPII thus facilitate assembly of other factors of polyadenylation machinery
	64	CstF 2	Interact with G/U or U-rich downstream signals
	77	CstF 3	Connect CstF-64 and CstF-50 and interacts with CPSF1
CFIm (cleavage factor Im complex)	25	NUDT21 (CPSF5)	Interact with upstream element (USE) containing U(G/A)UA, serves crucial role in 3' end processing
	68	CPSF6	
	59	CPSF7	
CFIIm (cleavage factor IIm complex)	-	PCF11	Unknown

Adapted from: (Colgan and Manley 1997, Zhang et al. 2005, Sagawa et al. 2011, Charlesworth et al. 2013).

The most commonly found nucleotide followed next to cleavage site is cytosine (Tian and Manley 2013). Upon transcription at the poly (A) site by RNAPII CPSF, which was initially linked with the CTD, is recruited to poly (A) signal leading to RNA cleavage and termination of transcription (Glover-Cutter et al. 2008). Upon

recognition, the recruitment of CPSF and CstF subunits to their respective sequence elements on pre-mRNA is assisted by CFIm, together with RNAPII, CF IIm and PAP, forming a competent cleavage complex as shown in figure 1.2. After the assembly of multi protein complexes to their specific cis acting regulatory sites on pre-mRNA, cleavage occurs which is followed by addition of the poly (A) tail (Zhao et al. 1999).

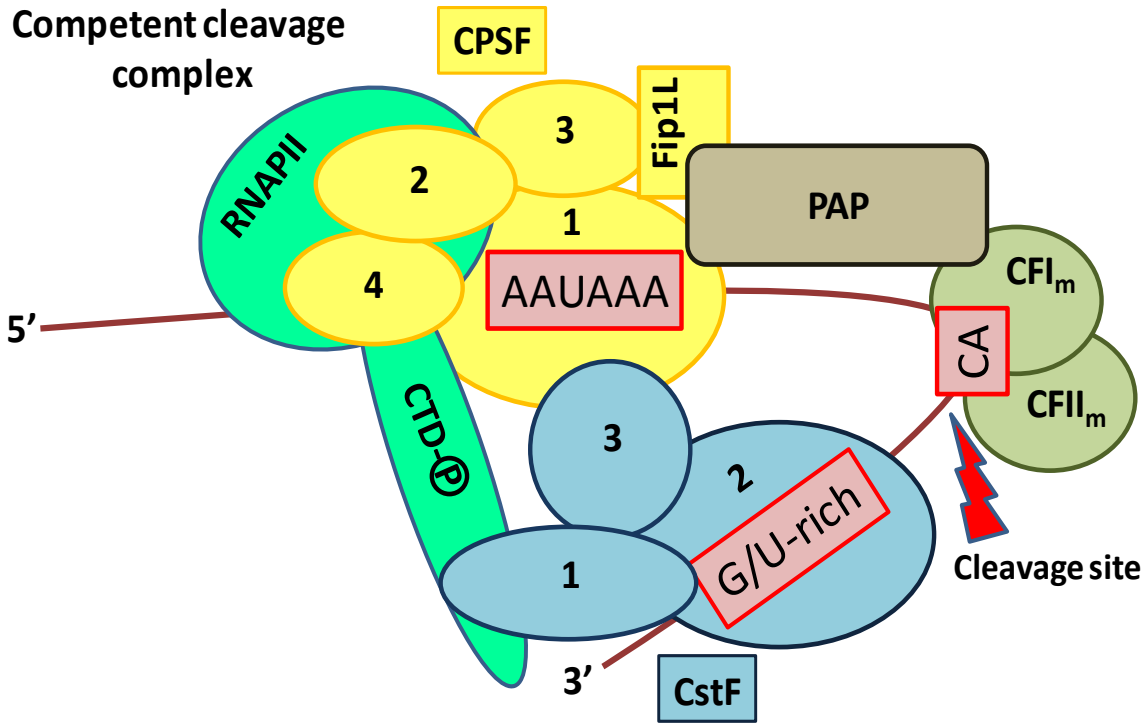


Figure 1.2: Schematic representation of factors involved in cleavage process:

Recruitment of protein factors of cleavage and polyadenylation machinery at their cognate sites on pre-mRNA for 3' end processing. CPSF 1(160) specifically recognises poly (A) signal which associates with PAP and is involved in connecting CstF 3 (77) and PAP. CstF interact with GU rich region by CstF 2 (64). CF Im and CF IIm are also required for efficient cleavage process.

Abbreviations: AAUAAA (poly (A) signal), CstF (cleavage stimulating factor), CPSF (cleavage/polyadenylation specificity factor), Pol II (RNA polymerase II), CTD (C-terminal domain), CF Im (cleavage factor I), CF IIm (cleavage factor II), PAP (poly(A)-polymerase).

Binding of PAP (poly (A)-polymerase) to RNA is weak and non-specific in the presence of Mn^{2+}, however in combination with Mg^{2+} and CPSF, PAP becomes specific for AAUAAA and the complex of PAP, CPSF, CstF and RNA is stabilized,

mediating polyadenylation (Zhao and Manley 1996). Soon after, the endonucleolytic cleavage nuclear PAP adds 200-250~ adenosine residues as shown in figure 1.3 (Zarkower and Wickens 1987, Meijer et al. 2007, Cevher et al. 2010), which is bound by PABPN1 (Nuclear poly A binding protein). The first poly(A) polymerase (PAP alpha or PAPOLA) isolated from calf thymus, is thought to be the main mediator of nuclear polyadenylation by most mRNAs (Bardwell et al. 1990). It has been reported that poly(A) polymerase is required for both cleavage and polyadenylation. A second canonical poly(A) polymerase, PAP gamma (PAPOLG) has also been linked to nuclear polyadenylation (Ghoshal and Jacob 1991, Zhao and Manley 1996, Zhao et al. 1999).

Except for PABP, all protein factors are necessary for in vitro cleavage, but for in vitro polyadenylation only CPSF (Fip1L), PAP and PABP are required (Mandel et al. 2008). Nuclear poly(A) binding protein (PABPN1) controls the final length of the poly(A) tail, by disrupting the interaction of CPSF (Fip1L) and PAP, as the poly (A) tail reaches the length of ~250 nucleotides by an unknown mechanism (de Klerk et al., 2012., Eckmann et al., 2011; Kuhn et al., 2009). The association of other proteins with polyadenylation machinery, such as nucleophosmin, plays a significant role in the regulation of length of poly (A) tail and in export of an mRNA from nucleus to cytoplasm (Sagawa et al. 2011). The binding of PABPN1 is weak and it is replaced by PABPC1 (cytoplasmic poly (A) binding protein) while still in the nucleus (Hosoda et al. 2006). After export, PABPC1 then interacts with eIF4G initiation complex bound to the cap of the mRNA, resulting in the formation of the competent closed loop complex and initiation of translation (Danckwardt et al. 2008).

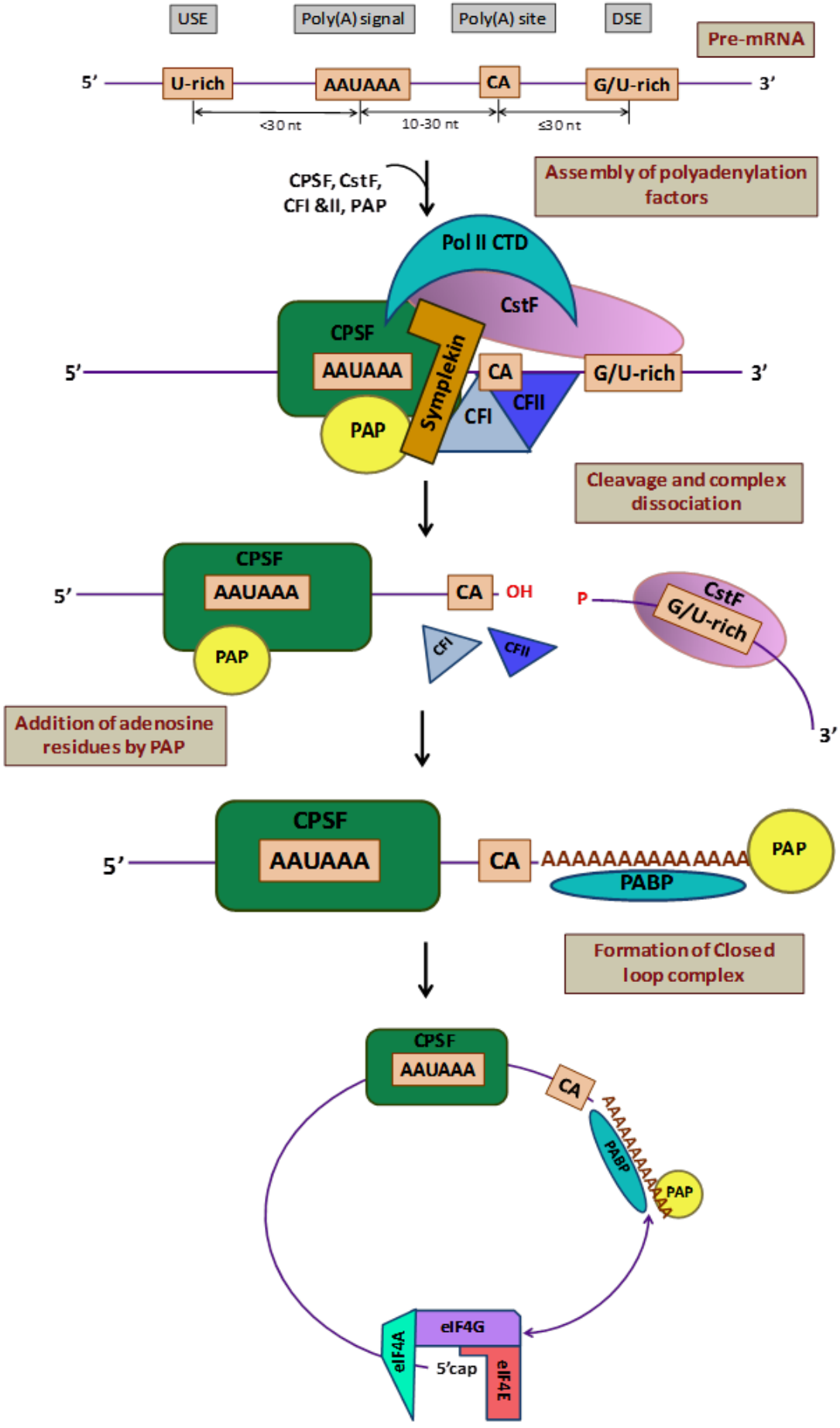

USE
Poly(A) signal
Poly(A) site
DSE
Pre-mRNA
5'
U-rich
AAUAAA
CA
G/U-rich
3'
<30 nt
10-30 nt
≤30 nt
CPSF, CstF,
CFI &II, PAP
Assembly of polyadenylation factors
Pol II CTD
CstF
CPSF
AAUAAA
Symplekin
CA
G/U-rich
PAP
CFI
CFII
Cleavage and complex dissociation
OH
P
Addition of adenosine residues by PAP
AAAAAAAAAAAAAA
PABP
Formation of Closed loop complex
eIF4G
eIF4A
eIF4E
5'cap

Figure 1.3: Process of nuclear cleavage and polyadenylation in eukaryotes:

Once the *Trans*-acting multiprotein complexes are assembled on their respective *cis*-acting RNA sequence elements, the pre-mRNA is cleaved endonucleolytically by CPSF-3 (CPSF73). This is followed by the addition of adenosine residues by PAP to form poly (A) tail, which is initially bound to PABPN1. Upon nuclear export PABPN1 is replaced by cytoplasmic PABPC. PABPC forms the closed loop ribonucleoprotein complex upon interaction with translation initiation factor eIFG, a part of the translaton initiation complex.

Abbreviations: USE (upstream sequence element), AAUAAA (poly(A) signal), DSE (downstream sequence element), CstF (cleavage stimulating factor) (blue complex), CPSF (cleavage/polyadenylation specificity factor) (light blue complex), Pol II (RNA polymerase II), CF I (cleavage factor I) (green complex), CF II (cleavage factor II), PAP (poly(A)-polymerase), PABPN1 (nuclear poly(A)-binding protein), PABPC (cytoplasmic poly(A)-binding protein) 4A, 4E, 4G (translation initiation factors).

1.3.2 Regulation of gene expression by co-transcriptional processing of the 3' end of an mRNA

Formation of mRNA by RNAPII is linked with the recruitment of essential 3' end processing factors, located at the place of RNA synthesis in the nucleus (Zhao et al. 1999, Glover-Cutter et al. 2008). Co-transcriptional recognition of regulatory factors involved in the process of cleavage and polyadenylation of nascent pre-mRNA, is mediated through interactions with RNAPII during transcription elongation. This provides a mechanistic explanation for the coupling of transcription and 3' end mRNA processing. The recruitment of polyadenylation factors to RNAPII and the type of promoter plays an important role in determining the efficiency of polyadenylation (Mapendano et al. 2010, sWang et al. 2010).

Eukaryotic DNA-dependent RNA polymerase (RNAPII) is initially recruited in a hypo-phosphorylated state to the promoter. It is composed of up to 12 polypeptides in which Rpb1 is the largest subunit and contains the catalytic activity (Hsin and Manley 2012). Nuclear polyadenylation is restricted to nascent transcripts of Pol II, by the interaction of cleavage and polyadenylation factors with the C –terminal domain (CTD), a unique feature of RNAPII (Zhao et al. 1999). The carboxyl terminal domain (CTD) of the largest subunit Rbp1 of RNAP II is composed of conserved 52 heptapeptide repeats (N-Tyr^{1}-Ser^{2}-Pro^{3}-Thr^{4}-Ser^{5}-Pro^{6}-Ser^{7}-C) in humans (Proudfoot and O'Sullivan 2002, Ryan et al. 2004, Glover-Cutter et al. 2008, Proudfoot 2011, Hsin and Manley 2012, Mayer et al. 2012) and each repeat consist of three serine

residues reversibly phosphorylated throughout the process of transcription (Moore and Proudfoot 2009). The CTD determines the efficiency of all processing steps required for the formation of mature RNA, and its function is modulated by the different states of phosphorylation with the aid of kinases and phosphatases (Zhao et al. 1999, Hsin and Manley 2012). The CTD interacts with cleavage and polyadenylation factors and recruits them to RNAPII, especially if it is Ser 2 phosphorylated. In this way, RNAPII functions as part of the polyadenylation machinery (Yonaha and Proudfoot 2000, Proudfoot 2011, Hsin and Manley 2012).

The phosphorylation on serine 5 (Ser5P) by TFIIH associated kinase Cdk7 (cyclin dependent kinase-7) is involved in early stages of elongation (Moore and Proudfoot 2009). Phosphorylation on serine 2 (Ser2P) by Cdk9 (Cyclin-Dependent kinase-9) or Cdk12 (Davidson et al. 2014) during late stages of transcriptional elongation recruits the cleavage and polyadenylation complex to the poly (A) signal through CTD-interacting domains (CIDs) mediating 3'end formation of mRNAs, while polyadenosine binding protein (PABP) binds to the newly formed poly-A sequence (Shim et al. 2002, Glover-Cutter et al. 2008, Mapendano et al. 2010, Mayer et al. 2012). Ser5 is dephosphorylated by phosphatases during transcripton elongation, forming a pattern of phosphorylation in which Ser5-P is high at the start of transcription, while Ser2-P emerges at later stages of transcription.

The kinase activity of CDK9 (P-TEFb) and Cyclin T plays a regulatory role in transcriptional control of the elongating polymerase (Garriga and Grana 2004, Moore and Proudfoot 2009). A component of CFII, Pcf11 in mammals, has a CID (CTD interacting domain) and interacts with the Ser-2 phosphorylated CTD. This leads to recruitment of the polyadenylation complex, including CPSF1 and PCF11, to RNAP II as shown in figure 1.4 (Kuehner et al. 2011, Nagaike and Manley 2011). The phosphorylation of TFIIB serine residue 65 is important in the recruitment of CPSF and CstF to the promoter, just after the Ser5P phosphorylation of CTD RNAPII and before the initiation of transcription (sWang et al. 2010).

The last stages of co-transcriptional processing of an mRNA are important for preventing the formation of abnormal transcripts (Moore and Proudfoot 2009). For

efficient transcription, termination by RNAPII cleavage of an mRNA is a necessary event (Moore and Proudfoot 2009, Proudfoot 2011). According to the torpedo model, endonucleolytic cleavage of pre-mRNA at the poly (A) signal during transcription is an essential step required for exonucleases activity of Xrn2 (Rat1 in yeast). Xrn2 is thought to attach to the free 5' end generated by polyadenylation-associated cleavage and contacts RNAP II, resulting in conformational changes and leading to the dissociation of nascent RNA from Pol II. Thus it leads to arrest of the elongation complex of Pol II and favours termination (Rosonina et al. 2006, Proudfoot 2011). The ability of polyadenylation factors such as Pcf11 to bind to the CTD is indeed a prerequisite for transcription termination (Hsin and Manley 2012).

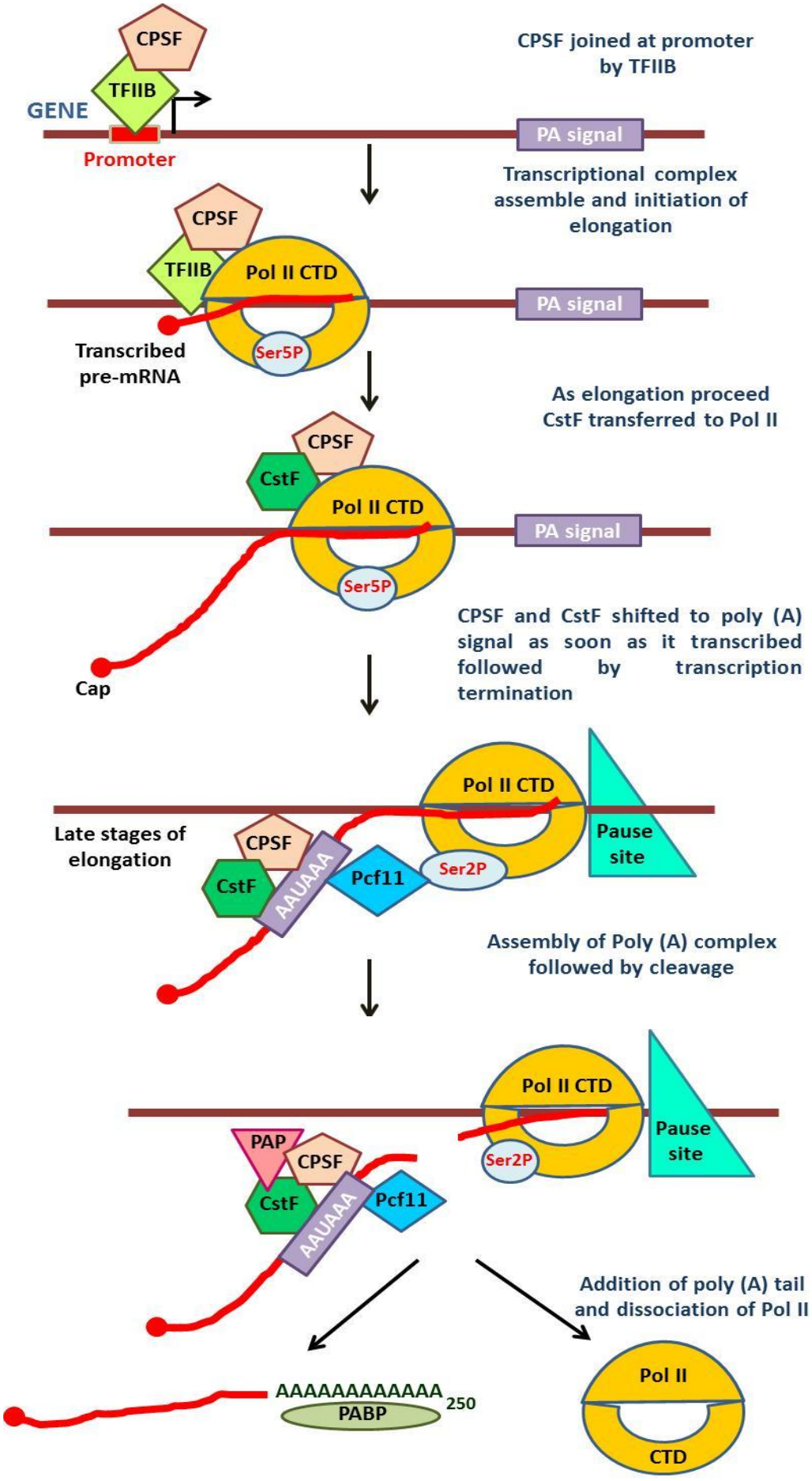

CPSF
TFIIB
GENE
Promoter
CPSF joined at promoter by TFIIB
PA signal
Transcriptional complex assemble and initiation of elongation
CPSF
TFIIB
Pol II CTD
PA signal
Ser5P
Transcribed pre-mRNA
As elongation proceed CstF transferred to Pol II
CPSF
CstF
Pol II CTD
PA signal
Ser5P
Cap
CPSF and CstF shifted to poly (A) signal as soon as it transcribed followed by transcription termination
Pol II CTD
Pause site
Late stages of elongation
CPSF
CstF
AAUAAA
Pcf11
Ser2P
Assembly of Poly (A) complex followed by cleavage
Pol II CTD
Pause site
Ser2P
PAP
CPSF
CstF
AAUAAA
Pcf11
Addition of poly (A) tail and dissociation of Pol II
AAAAAAAAAAAA
250
PABP
Pol II
CTD

Figure 1.4: Mechanism of transcriptional coupled polyadenylation:

Polyadenylation factor CPSF is initially recruited to the promoter by TFIIB, a part of PIC, which is later transferred to the transcription complex of CTD (Ser5P) RNAP II at elongation initiation stage. As elongation proceeds, CstF is transferred to CTD RNAP II. As soon as the RNAP II transcribes the poly (A) signal, cleavage and polyadenylation factors are recruited on newly transcribed mRNA from the CTD (Ser2P) of RNAPII. Thus upon recognition of the poly(A) signal, transcriptional elongation pauses just next to the poly(A) signal, resulting in termination of transcription, leading to the cleavage of RNAPII associated 3'end of an mRNA. This in turn leads to the addition of poly (A) tail of ~200-250 residues by PAP bound by PABP. Assembly of poly (A) complex leads to the cleavage of an mRNA, which expels the CTD results in dissociation of pause Pol II from the template.

Abbreviations: AAUAAA (poly (A) signal), CstF (cleavage stimulating factor), CPSF (cleavage/polyadenylation specificity factor), Pol II (RNA polymerase II), PAP (poly (A)-polymerase), PABP (Poly (A)-binding protein). TFIIB (Transcription factor IIB), CTD (C terminal domain).

Two possible mechanisms of transcriptional termination coupled to cleavage and polyadenylation have been reported. In most genes, a transcriptional pause site is located just next to the poly(A) signal at which cleavage occurs, while Pol II is still involved in transcription. (Zhao et al. 1999). Another class of termination occurs in the absence of a pause site downstream to the poly(A) signal, causing RNAPII to move further down the 3' flanking region of an mRNA. An additional 3' flanking termination sequence is found further downstream, which is known as the cotranscriptional cleavage (CoTC) sequence. The CoTC mediates a downstream cleavage event that precedes cleavage of the polyadenylation site. The degradation of nascent transcripts by Xrn2 at the 3' flanking region results in Pol II dissociation from DNA templates along pre-mRNAs, which are still linked to the CTD via poly(A) complexes (Moore and Proudfoot 2009, Proudfoot 2011).

1.3.3 Alternative polyadenylation

In alternative polyadenylation (APA) a gene possesses multiple poly(A) sites which result in the generation of different mRNAs, usually producing a variety of mRNAs with different 3' UTRs. More than 50% of protein encoding mammalian genes are subject to APA (Nunes et al. 2010). Alteration in length of the 3'UTR can change the presence of binding sites for miRNA or protein factors involved in maintaining the stability of an mRNA and in gene specific regulation as shown in figure 1.5 (de Klerk et al; 2012). The efficiency of the cleavage and polyadenylation processes

varies and selection of stronger downstream polyadenylation signals is thought to result from the ineffficiency of weaker upstream signals (Colgan et al. 1998). Shorter 3' UTRs, presumably generated through recognition of weak upstream APA sites in the presence of higher levels or activity of the polyadenylation machinery, have been observed to be enriched in cancer (Sandberg et al. 2008, Mayr and Bartel 2009). These shorter 3' UTR are closely associated with malignant transformation compared to rates of cellular proliferation (Sagawa et al. 2011). How APA is regulated in cancer is still unclear (Weill et al. 2012, Tian and Manley 2013). The machinery of cleavage and polyadenylation, splicing and transcriptional factors, have all been implicated in the regulation of APA (de Klerk et al., 2012; Proudfoot, 2011).

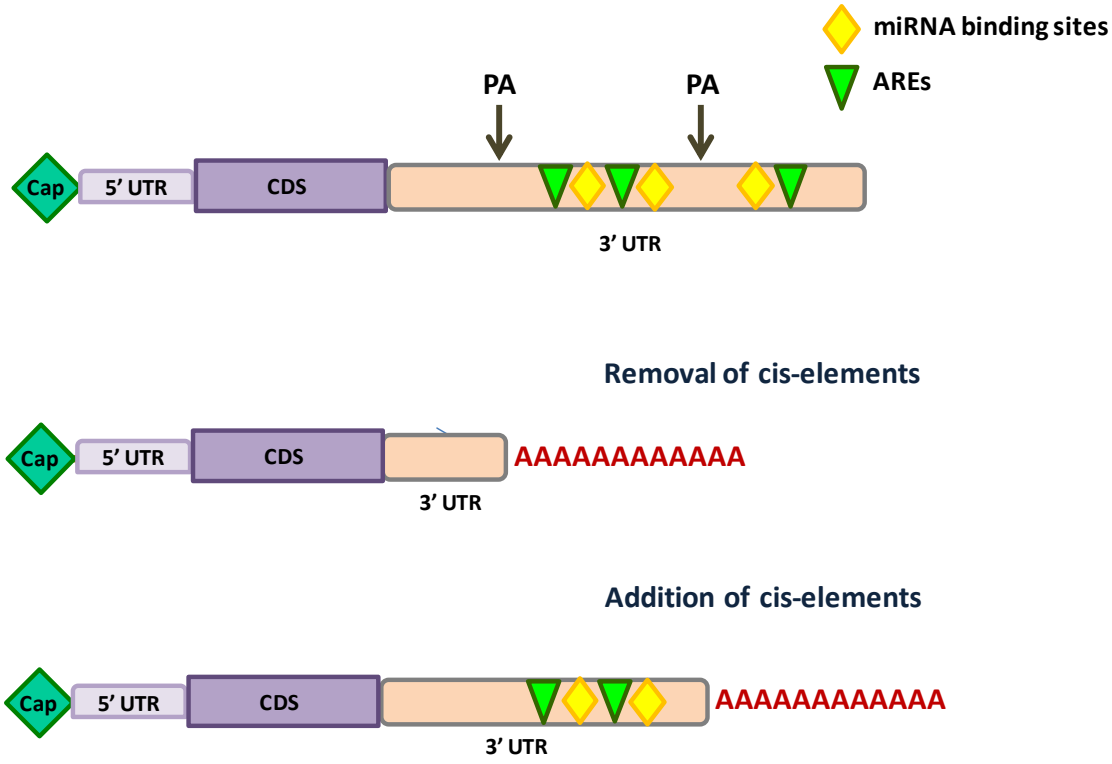


Figure 1.5: Diagrammatic representation of a gene containing cis-acting elements with APAs.

Gene containing two sites for alternative cleavage and polyadenylation located on 3' most exon of gene generates variable 3' UTR. Due to usage of different APAs an mRNA generates a transcript with or without the regulatory elements such as miRNA binding sites and AU-rich elements. PA: Poly (A) site, CDS: coding sequence, UTR: untranslated region.

The shorter mRNA isoforms are generally much more stable and are well translated. In many cases, loss of miRNA binding sites on the 3' UTR is probably involved in this effect (Mayr and Bartel 2009, Elkon et al. 2012).

1.3.3.1 Alterations in trans-acting factors

An example of regulation of APA is alterations in the levels of the CstF 64-kDa subunit, which lead to the selection of alternative poly(A) sites. During B cell differentiation, CstF-64 regulates the processing of pre-mRNA of the IgM heavy chain (μ). Increased levels of CstF-64 upon B-cell activation, results in a change of mRNA expression of the membrane bound form (μm) of IgM (μ) heavy chain to the secreted form (μs). This is due the selection of the upstream poly (A) site specific for μs. Furthermore, a decrease in the levels of CstF 64 in B cells results in cell cycle arrest in G_0/G1 phase, while its depletion results in apoptotic cell death. The process of alternative polyadenylation also affects many mRNAs in LPS stimulated macrophages and this also is a consequence of upregulation of CstF-64 expression. The amount of CstF-64 in cells not only involves in regulation of alternative polyadenylation but also plays an important role on level of RNA by modulating the gene expression (Takagaki and Manley 1998, Danckwardt et al. 2008).

PABPNI (poly A binding protein nuclear 1) not only plays an important role in the basal polyadenylation but changes in PABPNI expression also result in the selection of different alternative polyadenylation sites. The low levels of functional PABPN1 in OPMD (Oculopharyngeal muscular dystrophy), a muscular disorder, is caused by alternative polyadenylation site selection, which leads to altered gene expression (de Klerk et al. 2012).

1.3.4 Mechanism of cytoplasmic polyadenylation

During oogenesis and early development in animals, maternal mRNAs are stored in the cytoplasm of the oocyte in an under-adenylated state and become translationally activated by cytoplasmic polyadenylation at later stages of development (Weill et al. 2012). Therfore cytoplasmic regulation of polyadenylation requires particular factors involved in the shortening of poly(A) tail and in translation repression, in addition to the specific factors required for the induction

of cytoplasmic polyadenylation (Kojima et al. 2012, Charlesworth et al. 2013). The mechanism of cytoplasmic polyadenylation has been well studied in *Xenopus* oocytes. The best studied cases show that cytoplasmic polyadenylation requires two elements on its 3' UTR, the poly(A) signal bound by a cytoplasmic CPSF complex lacking the 73 kDa subunit, and a U-rich conserved cytoplasmic polyadenylation element (CPE) (UUUUUAU) bound to CPE-binding protein (CPEB1). Other cytoplasmic polyadenylation factors also exist (Fox et al. 1989, McGrew and Richter 1990, Gebauer and Richter 1996, Charlesworth et al. 2013).

Symplekin is a scaffold protein that binds CPSF, the non-canonical poly(A) polymerase Gld2 and CPEB. PARN, an enzyme that removes poly(A) tails, is also part of the CPEB complex (Zhang et al. 2010), as depicted in Figure 1.6. Cytoplasmic polyadenylation is induced by phosphorylation of CPEB1 by Aurora A (Richter 2007). This results in the expulsion of PARN from the complex (CPSF, Gld2 and symplekin), allowing Gld2 to elongate poly(A) tails and activates the process of translation (Novoa et al. 2010, Kojima et al. 2012). In oocytes, the poly(A) tail is bound by embryonic poly(A) binding protein (ePAB) (Zhang et al. 2010).

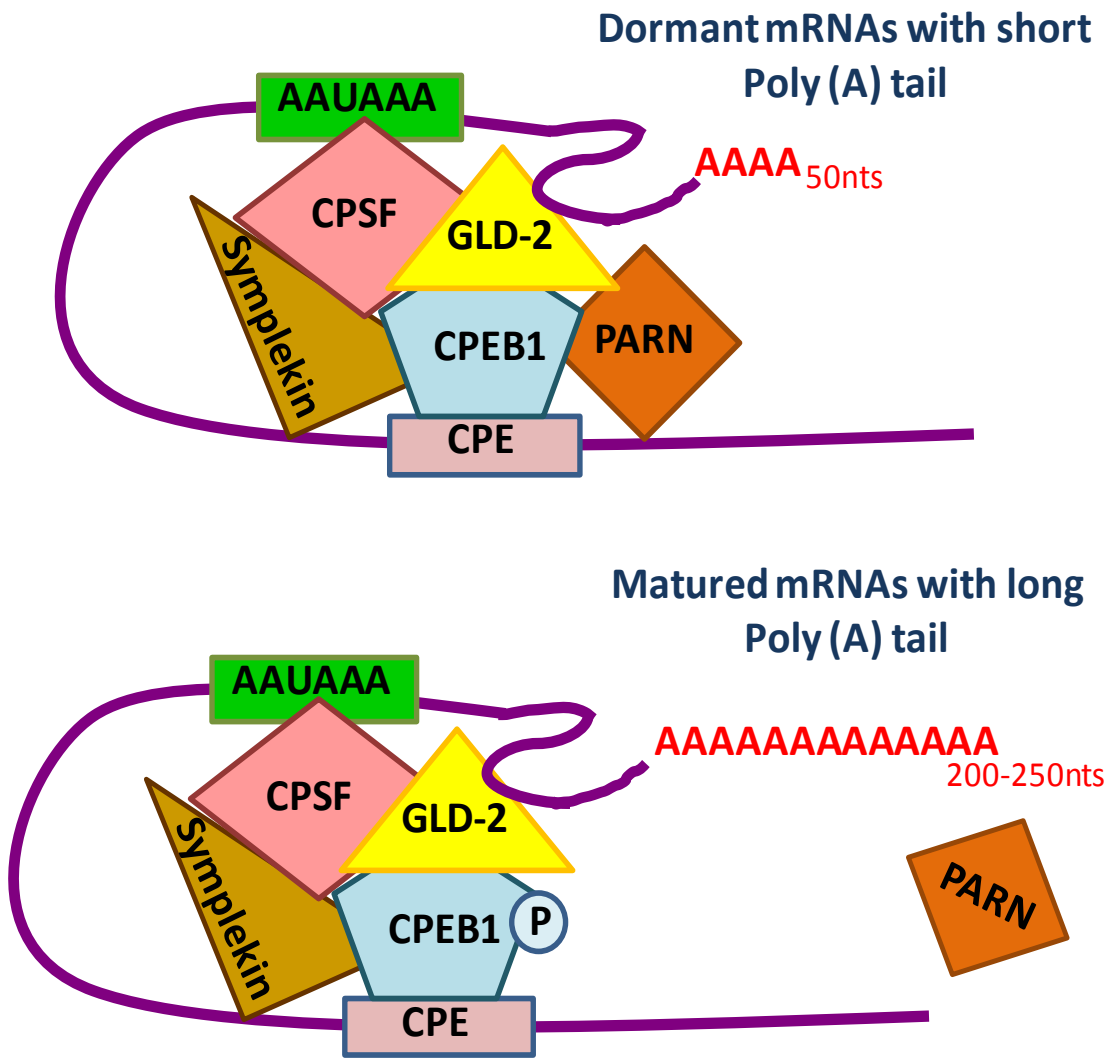


Figure 1.6: Regulation of poly (A) tail length by cytoplasmic polyadenylation:

Process of cytoplasmic polyadenylation involves the recognition of cis acting elements at 3'UTR such as CPE and AAUAAA (PA signal) by trans-acting factors CPEB and CPSF respectively. Complex of CPEB contains PARN and Gld2 which plays crucial role in poly (A) tail length regulation. By default mRNAs gets longer poly (A) tail in nucleus. The length of poly (A) tail after nuclear polyadenylation gets shorter upon cytoplasmic export due to deadenylation by PARN. CPEB, CPSF, PARN and Gld2 are associated together in mRNP complex assembled by the scaffold protein symplekin. Gld2 remains inactive until the progression of meiotic cell division or upon oocytes maturation which phosphorylates CPEB leads to dissociation of PARN from CPEB complex. Gld2 remains no longer restricted leads to the elongation of poly (A) tail followed by translation of specific mRNAs.

Abbreviations: CPE: cytoplasmic polyadenylation element, CPEB: CPE- binding protein, CPSF: cleavage-polyadenylation and specificity factor, Gld2: Germline development 2, PARN: Poly (A)-specific ribonucleases, ePAB: Embryonic poly (A) binding, mRNP: protein ribonucleoprotein.

1.4 Role of Poly (A) polymerases in polyadenylation

1.4.1 General characteristics of poly (A) Polymerase

The first poly(A) polymerase (PAP) was isolated from calf thymus in the beginning of the 1960's (Edmonds and Abrams 1960, Mandel et al. 2008). PAP is the enzyme that covalently adds the 3' poly (A) tail of mRNAs (Zhao and Manley 1996, Martin et al. 2008). PAP activity can be mediated by a variety of enzymes and is present in prokaryotes as well in eukaryotes. In eukaryotes it is present in mitochondria, nucleus, RNPs (ribonucleoprotein particles) and ribosomes (Jacob et al. 1989). PAPs play diverse roles from marking aberrant nuclear transcripts to stabilizing the small non coding RNAs (miRNAs) in cytoplasm (Katoh et al. 2009, Shcherbik et al. 2010). Addition of the poly(A) tail to 3' ends of an mRNA by PAPα in the nucleus is carried out in a processive manner until it reaches a length of ~250 residues (Martin et al. 2004).

1.4.2 Structure of poly (A) polymerases

PAPs belong to the superfamily of the template-independent RNA specific nucleotidyl transferases (rNTrs), which are responsible for the covalent addition of ribonucleotides as part of 3'end mRNA processing. Members of rNTrs and PAPs share a similar active site structure with that of the template dependent DNA polymerase β (Pol β), a DNA repair enzyme, and have sequence similarity to other enzymes in their catalytic domains, such as RNA and DNA polymerases (Martin and Keller 1996, Zhao et al. 1999, Martin et al. 2004, Martin and Keller 2007, Schmidt and Norbury 2010). The nucleotide substrates of rNTRs are a wide range of substrates, from single AMP or UMP residues to the addition of the trinucleotide CCA to tRNA at 3'ends (Martin et al. 2008). Other members of this group mediate the addition of single to several residues of UMP, these are known as TUTases (terminal uridylyl transferases) (Martin and Keller 2007). The majority of PAPs are conserved among eukaryotes (Zhao et al. 1999).

PAPs in general consist of three main domains: the catalytic domain found near the N terminal, followed by the central and the RNA binding domain near C-terminal NLS (nuclear localization signal), which overlaps with the RNA binding domain

(Balbo and Bohm 2007). All canonical PAPs share similar catalytic domains at the N terminal, which is connected to the RNA binding domain by the central domain. The nuclear localization signal (NLS) overlaps with the RNA binding domain. The C-terminal domain (residues 354–530) of canonical PAPs is responsible for binding the cleavage/polyadenylation subunit Fip1, in which three constant aspartate residues interact with two metals out of three metals of the active site, among which one interacts with the adenine ring. This interaction serves a crucial role in structural selection of ATP by poly(A) polymerase (Martin et al. 2000). Addition of ribonucleoside monophosphate on RNA 3' OH group by PAP is accomplished by two metal ions in the active site. One binds to RNA while the other one interacts with the phosphate group of the incoming nucleotide and the 3' OH group of a pre-mRNA. PAPα is highly specific for ATP selection (Martin and Keller 2007). Identification of substrate and nucleotide selection by PAPα is due to the induced fit mechanism of enzyme (Martin and Keller 2007, Mandel et al. 2008).

1.4.3 Types of PAPs

The PAPs are classified into canonical and non canonical on a structural and functional basis in table 4. The eight identified PAP genes in mammals are PAPOLA (pap α), PAPOLB (pap β), PAPOLG (pap γ), PAPD1, PAPD2, PAPD4, PAPD5, and PAPD7 (PAP associated domain containing 1, 2, 4, 5, 7 respectively) (Wahle and Ruegsegger 1999, Kashiwabara et al. 2008). PAPs α, β and γ were first identified in mammals, are found in nucleus and are now called canonical PAPs. PAPD4 (GLD-2) (germline development gene), Trf-4/5, PAPD5, MtPAP and POLS were identified later so are known as non canonical rNTrs (Martin and Keller 2007).

GLD-2, Trf-4/5 and other non-canonical PAPs contain a similar catalytic domain to canonical PAPs, but differ in their nucleotide recognition motifs. Non canonical PAPs lack the RRM-like RNA binding domain. TUTase and Mt PAP have different potential RNA binding domains in their N terminus, as indicated in figure 1.7. Most non-canonical PAPs therefore require additional protein factors which recruit them to their target RNAs.

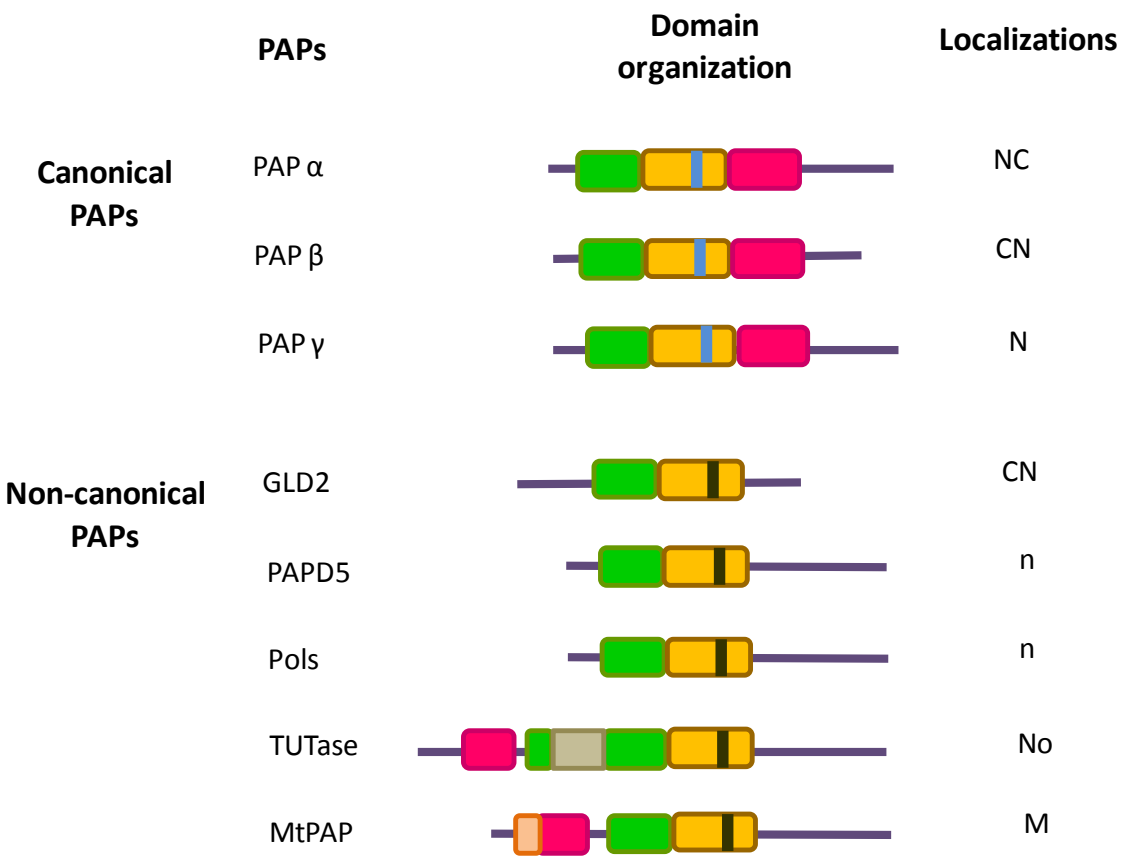


Figure 1.7: Structural organization of types of RNA specific nucleotidyl transferases:

Three canonical and five non canonical PAPs are shown. Localizations in cells are indicated as C (cytoplasmic), N (nuclear), M (mitochondria) and No (nucleolar). Domains are indicated as: green for catalytic domain, Orange for central domain, pink for RNA binding domain, blue for nucleotide recognition motif in canonical PAPs (NRM type1), dark brown for NRM type 2 in non canonical PAPs, light brown or Tan in U6 TUTase.

Trf4 and Trf5 were initially identified in *S.cerevisiae* as being part of the TRAMP complex and are involved in transcript quality control and decay in the nucleus (Shcherbik et al. 2010). RNA oligoadenylated by the TRAMP complex becomes a substrate for degradation by the nuclear exosome. In addition to Trf4/5, the TRAMP complex consists of the RNA helicase (Mtr4p) along with RNA binding proteins Air1 and Air2. Complete turnover of RNA requires multiple rounds of oligoadenylation (by the TRAMP complex) of intermediates degraded by 3' exosome (Martin et al. 2008).

Table 4: Types of mammalian poly (A) polymerases

Types of PAPs	Synonyms/homologs	Characteristics
PAP-alpha (Papα)	PAPOLA, PAPII	Found predominately in nucleus adds poly (A) tail to the end of an mRNA (Bardwell et al. 1990). Also involved in some cases of cytoplasmic polyadenylation (Hwang et al. 2001, Radford et al. 2008). Different isoforms as a result of APA have been reported. PAP interacts with CPSF-1 and Fip1L by its C-terminal region (Martin and Keller 2007).
PAP- beta (Papβ)	PAPOLB, TPAP or PAPT	Testis-specific cytoplasmic PAP, mediates in polyadenylation of mRNA transcripts for spermatogenesis (Benoit et al. 2008, Kashiwabara et al. 2008). papβ is found in cytoplasm and nucleus both (Lee et al. 2000).
PAP-gamma (Papγ)	PAPOLG, Neo-PAP, SRP RNA 3'-adenylating enzyme	Involved in 3'end processing of pre-mRNA by polyadenylation, interacts with components cleavage and polyadenylation machinery (Martin and Keller 2007). Found in nucleus, very similar to PAPα (Topalian et al. 2001). Is 60-75% homologous to amino termini region of PAPα. Also mediates mono adenylation of small RNAs at 3' end such as U2 small nuclear RNA, signal recognition particle (SRP) RNA, ribosomal 5S RNA and nuclear 7SK RNA (Kyriakopoulou et al. 2001, Perumal et al. 2001).
Gld2	PAP associated domain containing 4 (PAPD4)	Mediates cytoplasmic polyadenylation of CPE containing specific mRNAs upon oocytes maturation in *Xenopus* as well in *Drosophila* embryo and also in neurons (Radford et al. 2008). Regulate transition from mitosis to meiosis. Initially identified in *C. Elegans* (Stevenson and Norbury 2006), where it forms a complex with RNA binding protein Gld3 which is recruited it to specific mRNAs (Martin and Keller 2007). Also stabilizes miRNAs by 3' end monoadenylation (D'Ambrogio et al. 2012). Involved in spermatogenesis and oogenesis in frog and mice as well in cytoplasmic polyadenylation in somatic cells. XGLD2 an ortholog of GLD-2 in *Xenopus* found to localized in nucleus and cytoplasm. *Drosophila* GLD2 limited to cytoplasm only (Rouhana et al. 2005, Schmidt and Norbury 2010).

Papd5	Gld4, Trf4 or 5p (topoisomerase-related function protein)	Involves in RNA quality control, oligoadenylates RNA by targeting exosome mediated cryptic RNA degradation, component of TRAMP complex, ortholog of *S. Cerevisiae,* found in nucleus (Fasken et al. 2011, Schmidt et al. 2011). Also involves in polyadenylation of specific mRNAs (Ciais et al. 2008, Houseley and Tollervey 2009, Rammelt et al. 2011). Found to involved in cytoplasmic polyadenylation in the absence of GLD-2 in *C. Elegans* and expressed in germ cells (Schmid et al. 2009). Role in histone mRNAs uridylation and degradation also involves in turnover of pre-rRNA in nucleus (Schmidt and Norbury 2010, Schmidt et al. 2011). In human fibroblast, it is required for CPEB1 mediated polyadenylation of *TP*53 mRNA (D'Ambrogio et al. 2013).
Pols (DNA polymerase sigma)	Trf4/PAP2/ PAPD7	RNA quality control, oligoadenylates RNA by targeting exosome mediated cryptic RNA degradation, component of TRAMP complex, ortholog of *S. Cerevisiae,* found in nucleus (Stevenson and Norbury 2006). Also involves in polyadenylation of specific mRNAs (Ciais et al. 2008, Rammelt et al. 2011).
Starpap	TUTase, Papd2	Eukaryotic specific U6 snRNA TUTase (terminal RNA uridylyl transferases) which add U residues at 3'end of an mRNA (Trippe et al. 2003, Trippe et al. 2006). Role in nuclear phosphoinositides mediated signalling pathways where it polyadenylates specific mRNAs (Schmidt and Norbury 2010).
Mtpap (mitochondrial poly(A) polymerase)	Mitopap, Papd1	Involves in polyadenylation of transcripts encoded in mitochondria, localize in mitochondria (Nagaike et al. 2008, Chang and Tong 2012). It also oligouridylate 3'ends and degrade histone mRNAs in cytoplasm (Bai et al. 2011).

1.5 Role of polyadenylation in cancer

1.5.1 Polyadenylation in cancer

Different factors of cleavage and polyadenylation machinery have been found to be elevated in cancer (Morris et al. 2012). High levels of the scaffold protein symplekin were observed in lung and colon cancer (Singh et al. 2009, Buchert et al. 2010, Cappell et al. 2010). High PAPOLA activity is correlated with cancer (including breast

cancer) (Scorilas et al. 2000) and high CPSF4 levels have been observed in lung cancer (Chen et al. 2013), indicating that changes in polyadenylation may play a role in tumorigenesis.

In addition, high levels of CPEB4 have been linked with aggressive and highly malignant forms of cancer such as in prostate cancer and promote metastasis through EMT (epithelial–mesenchymal transition) (Xu and Liu 2013). Upregulation of CPEB4 has also been observed in pancreatic ductal adenocarcinomas (Sosef and Dauby 2012).

Reduced levels of polyadenylation factors under oxidative stress and DNA damage have been reported during cell cycle arrest (Mirkin et al. 2008, Kiefer et al. 2009). Upon DNA damage, BRCA1 (breast cancer 1) associated protein BARD1, inhibits 3' end processing of an mRNA by interacting with CstF-50, thus inhibiting polyadenylation (Kleiman and Manley 1999, Scorilas 2002, Mirkin et al. 2008, Cevher et al. 2010). A specific point mutation in BARDI found in tumours leads to disruption of its association with the polyadenylation machinery (Thai et al. 1998).

1.5.1.1 Role of Poly (A) polymerases in cancer

PAP mutations in humans lead to cell arrest in G_0/G1 phase while overexpression is seen in human carcinomas such as breast, colon and ovary and in other haematological malignancies (Danckwardt et al. 2008). PAP gamma and PAP alpha are involved in nuclear polyadenylation and highly resemble each other. They were both found to be upregulated in tumour tissue (Topalian et al. 2001, Martin and Keller 2007). Hyperphosphorylation of PAP alpha by cyclin B1 during M phase leads to the inactivation of polyadenylation, which ultimately reduces the mRNA levels and protein production during mitosis (Colgan et al. 1996, Colgan et al. 1998). Therefore low levels of PAP result in cell cycle arrest and high levels of PAP correlate with less differentiated cells (Scorilas et al. 2000). Enhanced levels of PAP have been found in actively proliferating lymphocytes (Scorilas et al. 2000) and in colon cancer (Pendurthi et al. 1997). PAP has also been used as a biomarker in chronic leukaemia (Trangas et al. 1984). Upregulation of PAP has been observed in rapidly proliferating cells, such as in aggressive types of cancer (Scorilas et al. 2000),

increasing the stability of oncogene mRNAs and resulting in increased rounds of translation (Pendurthi et al. 1997).

1.5.2 Alternative polyadenylation

As discussed above, an increase in the use of proximal polyadenylation sites has been observed in proliferating cells and during oncogenic transformation (Elkon et al. 2012, Tian and Manley 2013). Different breast cancer cell lines such as transformed MCF-7 cells, exhibit shortening of 3' UTRs compared to non-transformed MCF10A, while MB231 shows lengthening of the 3'UTR (Fu et al. 2011).

1.6 Role of the Poly(A) tail in the life of an mRNA

Poly(A) tails play a major role in modulating the export, translation and stability of an mRNA (Kojima et al. 2012). The changes in the length of a poly(A) tail as a consequence of deadenylation is thought to be a determining factor of mRNA stability under different cellular and physiological conditions (Meyer et al. 2004, Zhang et al. 2010).

1.6.1 Poly(A) tails in mRNA stability

1.6.1.1 Deadenylation dependent decay of an mRNA

The steady state levels of an mRNA are a balance between mRNA turnover and mRNA synthesis (Cevher et al. 2010, Ma et al. 2013). mRNA stability can vary depending upon the mRNA. For instance, in mammalian cells the *c-fos* transcript undergoes a rapid decay with a half life of only 10-15 minutes in cytoplasm compared to *β*-globin transcript which is highly stable with a half life of several hours to 24 hours, depending on the species (Meyer et al. 2004). Two crucial modifications which mRNAs receive during their synthesis, are the 5' 7-methylguanosine cap and the poly(A) tail at 3'end. For an mRNA to fully decay, both or either modification has to be removed. The removal of the cap and the poly(A) tail are believed to be rate determining steps in mRNA decay (Meyer et al. 2004).

Poly(A) tails protect translationally active stable mRNAs from degradation, while aberrantly processed mRNAs are deadenylated leading to mRNA decay (Guhaniyogi and Brewer 2001, Goldstrohm and Wickens 2008, Eckmann et al. 2011, Chen and Shyu 2013). The decay of an mRNA is a biphasic process in nearly all eukaryotes and begins with a slow phase during deadenylation followed by rapid degradation of mRNA body, either by decapping or 5' and 3' exonucleases (Garbarino-Pico et al. 2007, Chen et al. 2008, Kuhn et al. 2009). Deadenylation is thought to be the rate determining and primary regulated step of mRNA degradation in mammalian cells. Pathways of mRNA decay via miRNA (microRNAs) or ARE (AU-rich elements) are also largely mediated through deadenylation (Chen et al. 2008, Chen and Shyu 2011).

The deadenylation of an mRNA begins with the initial shortening of the long poly(A) tail received in the nucleus by Pan2/Pan3 complex to ~30-60nts in mammals. This is followed by further reduction in length by aid of the cNOT complex to 8-10 nts depending upon the species, as shown in figure 1.8 (Chen and Shyu 2011). After deadenylation, the Lsm1-7 complex is recruited to the 3' end of an mRNA which induces decapping through DCP1/2 complex, in which DCP2 mediates cleavage of the 5' cap to m^7GDP leaving an mRNA containing a 5' monophosphate, while DCP1 initiates the hydrolysis of the cap followed by degradation of an mRNA body (Meyer et al. 2004). The degradation of an mRNA after deadenylation can be initiated in either direction. XRN1 degrades in the 5'-3' direction and the exosome degrades in the 3'- 5' direction, depending on the mRNA species (Schwede et al. 2009).

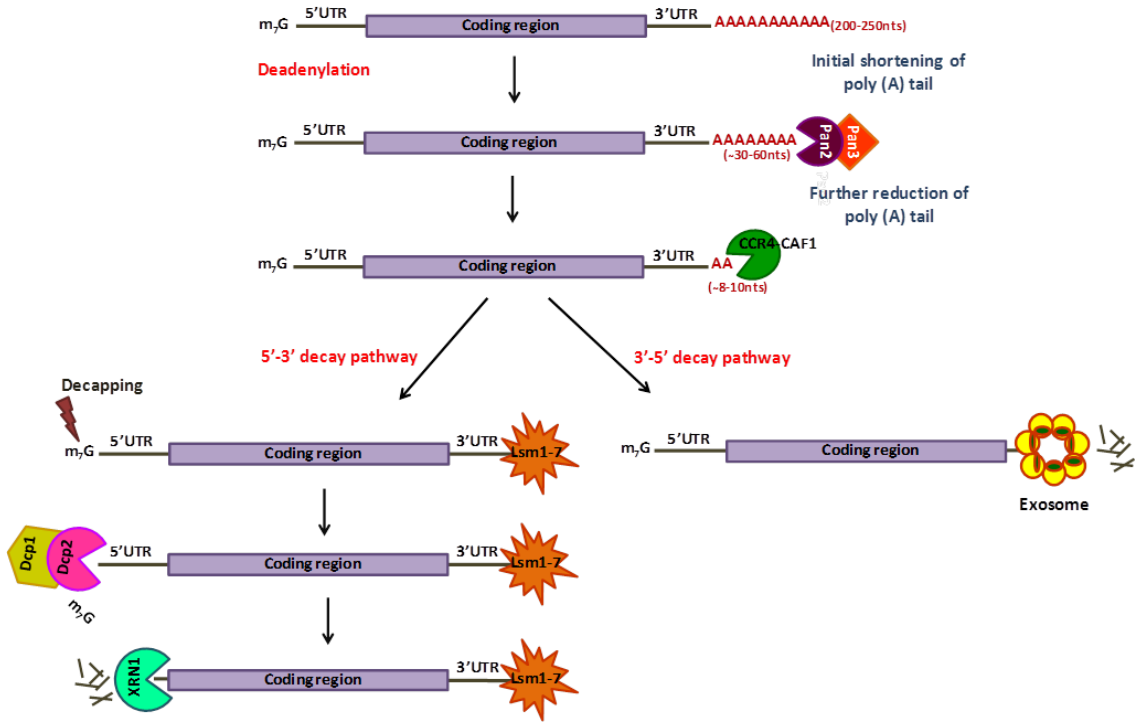


Figure 1.8: Schematic representation of deadenylation dependent decay of an mRNA:

In mammalian cells, deadenylation is accomplished in two phases. First initial shortening of poly (A) tail (~30-60nts) by Pan2/Pan3, followed by further shortening of poly (A) tail by cNOT complex upto 8-10 nts depending upon species. Decapping enzyme (Dcp1/2) is stimulated by Lsm1-7 complex attached at 3' end of an mRNA followed by decay of an mRNA body by XRN1 in 5'-3' direction. Alternatively, when 5'-3' pathway by XRN1 is compromised, mRNA can be degraded by the cytoplasmic exosome 3'-5' decay pathway.

1.6.2 Poly(A) tails in translation

1.6.2.1 Translational regulation:

1.6.2.1.1 Closed loop formation by the 5'cap and 3' poly(A) tail

The co-operative association between the 5'm^7G(5')PPP(5')N cap and the 3' poly (A) tail mediates translational initiation through the formation of the closed loop complex. This is the result of association of the translation initiation factor (eIF4F) complex through the 5' cap structure (Newbury 2006, Weill et al. 2012). The stability of the closed loop complex is a major determinant of translational regulation (Beilharz and Preiss 2009).

Translational regulation plays a key role in controlling major cellular activities including development, cell proliferation to differentiation in association with

several RNA binding proteins specific for various cis elements found on 5’ or 3’ UTR (Fukao et al. 2009). The process of translation is highly controlled and a rate determining step, which starts with the binding of translation initiation factors on the 5’ cap structure found on all nuclear encoded mRNAs (D'Ambrogio et al. 2013). In translation initiation, the cap binding eukaryotic translation initiation factor (eIF4F) is composed of eIF4A (an RNA helicase involved in RNA unwinding), eIF4E (directly binds to 5’ cap) and eIF4G, a scaffold protein which interacts with eIF4E and binds to multisubunit factor eIF3. eIF3 mediates the recruitment of the 40S ribosomal subunit (43S upon association with other key factors in pre-initiation complex) to the 5’ end of an mRNA (Fukao et al. 2009, D'Ambrogio et al. 2013).

The poly(A) tail enhances the efficiency of translational initiation through the interaction of eIF4F complex and PABP (poly(A) binding protein) bound to the 5’ cap and the 3’ poly(A) tail respectively (Fukao et al. 2009, Burgess and Gray 2010). The poly(A) tail of nuclear polyadenylated mRNAs is bound to PABPN1, which before cytoplasmic export is replaced by PABPC1 (PABP1 or PAB1) (Afonina et al. 1998, Burgess and Gray 2012). It promotes translation initiation by interacting with eIF4G, a subunit of translation initiation complex, which in turn binds to eIF4E, mediating the formation of the so-called closed loop structure as shown in figure 1.9 (Fukao et al. 2009, Burgess and Gray 2010, Brook and Gray 2012, Kojima et al. 2012). The association of PABPC with a protein similar to eIF4G, PAIP1 (PABP interacting protein 1), stabilizes the closed loop structure by enhancing the recruitment of eIF4A and eIF3 (Craig et al. 1998). The interaction of PABPC1 with eIF4A co factor, eIF4B, enhances the helicase activity of eIF4A, however its exact role needs further investigation (Cheng and Gallie 2007, Burgess and Gray 2010).

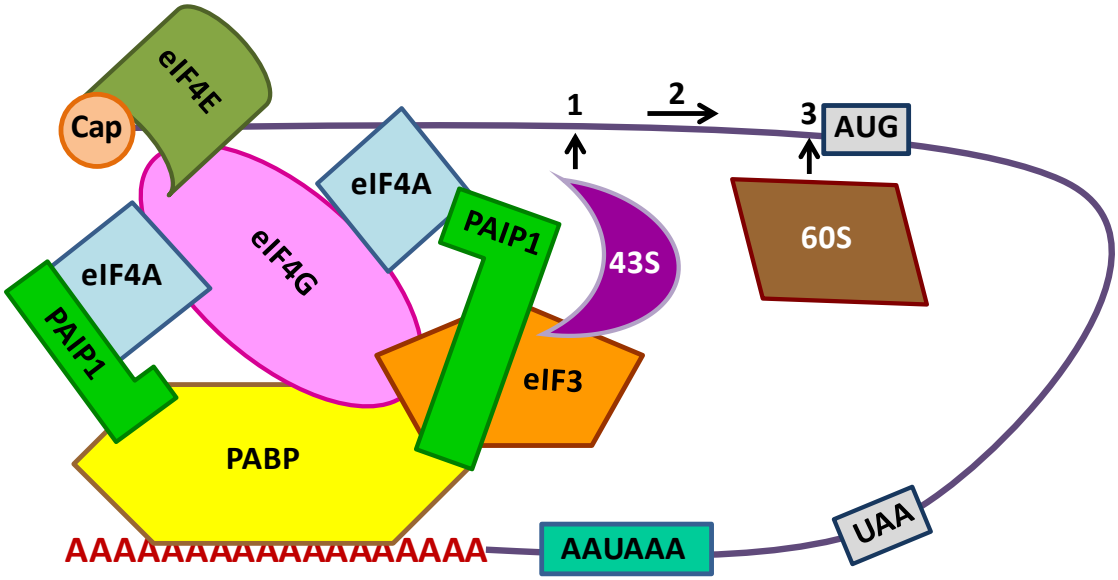


Figure 1.9: Role of PABP1 in translation initiation:

The poly(A) tail is bound by PABPC1 which connects the two ends of an mRNA by interacting with the eIF4F complex bound to the 5' cap resulting in formation of the closed loop circular structure. The components of eukaryotic translation initiation factor complex (eIF4F) are recruited to 5' cap. 1, the scaffold protein eIF4G interacts with eIF4E and binds to eIF4A and eIF3 which ultimately recruits 40S small ribosomal subunit (43S upon association with factors in PIC) at 5' end of an mRNA.

Abbreviation: ORF: open reading frame, PAIP1: PABP interacting protein 1.

1.6.2.1.2 Role of cytoplasmic polyadenylation in translational reactivation

The length of a poly(A) tail is also implicated in translational regulation, as cytoplasmic increases in poly(A) tail length correlate with translational activation of mRNAs. Upon specific stages of development, such as during oocyte maturation, the changes in poly(A) tail length and translational regulation of specific mRNAs are the consequence of deadenylation of long poly (A) tails made in the nucleus or the cytoplasmic polyadenylation of stored maternal mRNAs (Papin et al. 2008). Translational activation through cytoplasmic polyadenylation involves recruitment of translation initiation machinery by poly(A) tails through PABP, which results in dissociation of inhibitory factors bound to an mRNA (Radford et al. 2008). Freshly made mRNAs that have obtained a poly(A) tail in the nucleus are thought to translate more efficiently, while shorter poly (A) tails lead to poor translation of an mRNA (Kwak et al. 2008, Radford et al. 2008, Kuhn et al. 2009, Charlesworth et al.

2013). Therefore, length of a poly (A) tail is thought to be a major determinant of translation efficiency (Schmid et al. 2009, Weill et al. 2012), although a recent study indicates this may only hold true in early development. In any case, mRNAs require at least 25 nt to permit PABP binding and stimulation of translation (Schwede et al. 2009).

1.6.2.2 Role of deadenylation: translational repression

In general, poly(A) tail removal is the initial step in mRNA turnover or in silencing the cytoplasmic transcripts of specific mRNAs, but deadenylation does not always lead to mRNA decay. These stable deadenylated mRNAs can remain translationally inactive and become reactivated upon cytoplasmic polyadenylation (Weill et al. 2012). Deadenylation may mediate mRNA decay and translational repression by disrupting the association between translational initiation factors bound to the cap and PABP (Goldstrohm and Wickens 2008). The interaction between PABP and the translation termination factor eRF3 also stimulates deadenylation (Goldstrohm and Wickens 2008, Zhang et al. 2010). mRNA specific translational regulation controlling the fate of an mRNA, depends upon the special regulatory elements mostly found in 3'UTR. These cis-elements are bound by specific RNA binding proteins that assemble mRNAs into mRNPs (ribonucleoprotein complexes) (Schmid et al. 2009) and are involved in modulating the length of the poly(A) tail through recruitment of specific deadenylases (Goldstrohm and Wickens 2008, Weill et al. 2012). The interaction of translation components with the decay apparatus provides a mechanistic link with translational repression (Newbury 2006).

1.6.2.2.1 AU-Rich elements (ARE)

The AREs (AU-rich elements) are regulatory sequence elements in U-rich 3' UTR regions, which are recognised by various RNA binding proteins, which can prevent or promote decay as shown in figure 1.10. AREs are the best studied destabilizing elements and consist of pentamer repeats of 5'-AUUUA-3' found in the 3' UTR of many unstable eukaryotic mRNAs (Chen et al. 2008, Weill et al. 2012). AREs regulate approximately 3000 genes (Meisner et al. 2004) through their interaction with almost 30 ARE-binding proteins identified so far (Weill et al. 2012). Cytokines,

proto-oncogenes, transcription factors encoding transcripts and growth regulatory genes are highly enriched for AREs (Garneau et al. 2007, Meijer et al. 2007). AREs promote deadenylation-mediated decay through ARE-binding proteins such as TTP (tristetraprolin), KHSRP (KH-type splicing regulatory protein), BRF1 (butyrate response factor 1), ELAV (embryonic lethal abnormal vision) and AUF1 (AU-rich binding factor 1), which recruit deadenylating enzymes (Garneau et al. 2007, Zhang et al. 2010). AREs can also mediate stabilization through the only identified stabilizing ARE-binding protein HuR (Hu protein R), a member of the ELAV family (Meisner et al. 2004, Garneau et al. 2007, Zhang et al. 2010). The ARE-binding proteins TTP (Zfp36) and KHSRP recruit deadenylases, the cNOT complex and PARN respectively (Garneau et al. 2007, Weill et al. 2012).

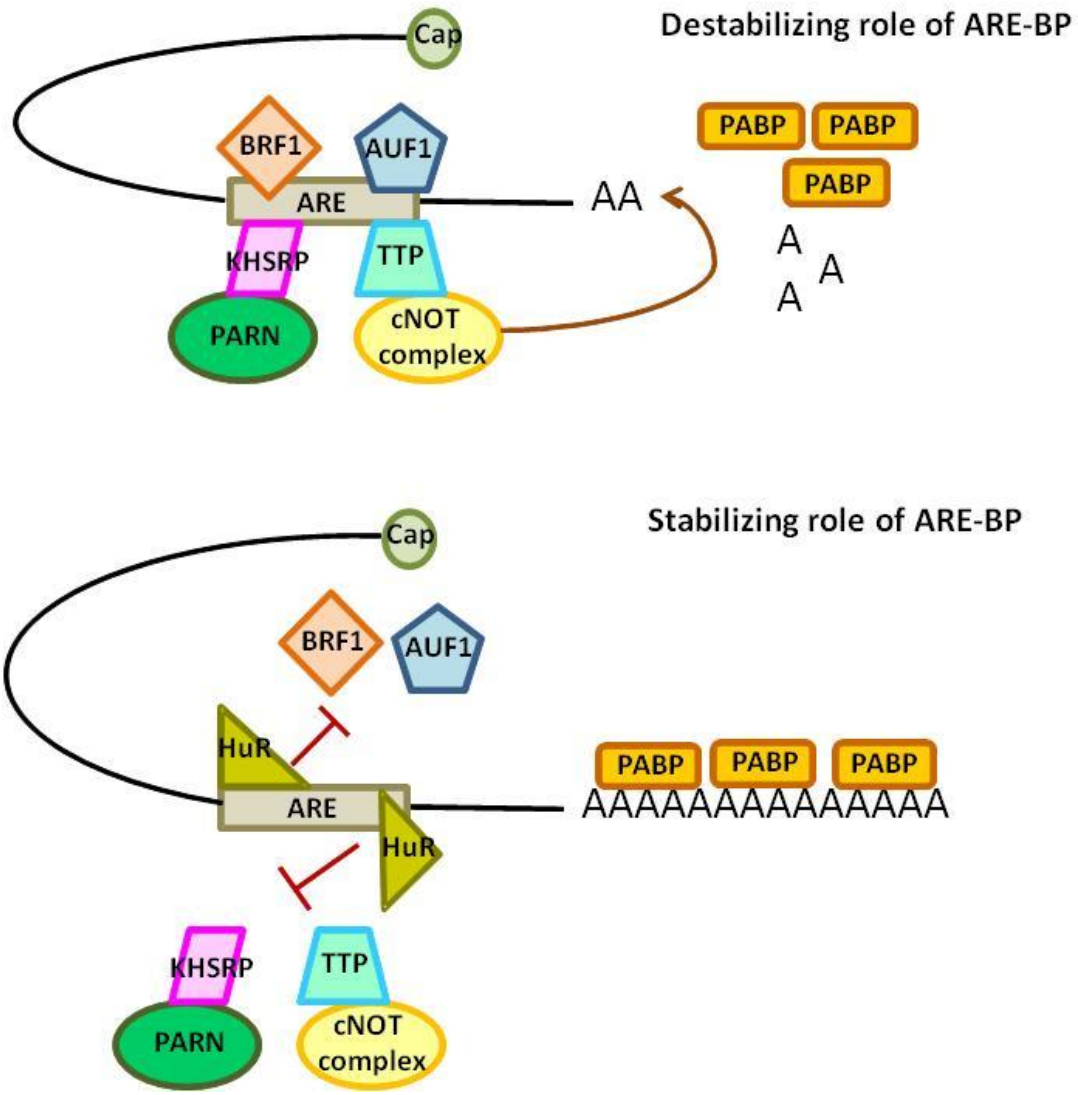


Figure 1.10: Regulation of deadenylation by AU-Rich elements:

AREs mediate deadenylation regulation through the recognition of several ARE- binding proteins which stabilize or destabilize of mRNA. AREs are recognized by many destabilizing factors such as AUF1, BRF1, KHSRP and TTP. KHSRP and TTP mediate recruitment of deadenylation enzymes such as the cNOT complex and PARN respectively. AREs containing mRNAs are stabilized through association of the ARE-binding protein HuR which acts as a positive regulator by preventing the interaction of destabilizing factors to AREs. HuR also stabilizes the interaction of PABPC with poly (A) tail.

Abbreviations: ARE (AU-Rich element), TTP (tristetraprolin), KHSRP (KH-type splicing regulatory protein), BRF1 (butyrate response factor 1) and AUF1 (AU-rich binding factor 1).

Adapted from: (Zhang et al. 2010).

1.7 Cordycepin: highly prized traditional Chinese medicine

1.7.1 History and habitat of the caterpillar fungi

Cordyceps militaris and *Ophiocordyceps sinensis* (formerly Cordyceps sinsensis) infect caterpillars before producing fruiting bodies. *O. sinensis* has been used medicinally in China and Tibet for more than 300 years. . It is called 'Dong- Chong Xia Cao' (winter worm summer grass) and was first mentioned by Wang Ang in 1694 in Ben-Cao-Bei-Yao (Yue et al. 2013). It is used for a large variety of diseases, including lung and kidney diseases and diseases of old age. *Cordyceps militaris* also parasitizes Lepidoptera larvae and is found throughout the Northern hemisphere, however fruiting bodies are very rare in all locations, in contrast to *O. sinensis,* which could be collected in quantity in Tibet (Cunningham et al. 1950, Kim et al. 2006, Imesch et al. 2012).

Collection of *Ophiocordyceps sinensis* is the source of 40% of cash income in rural Tibet. This is due to the high prices it fetches in China as the fungus is becoming rare in the wild (Winkler 2010). *Ophiocordyceps sinensis* does not form fruiting bodies in culture, and the level of cordycepin in cultivated samples is low (Utami, De Moor and Barrett, unpublished result). In contrast, *Cordyceps militaris* can be persuaded to form fruiting bodies in culture and strains with very high cordycepin production have been selected. All cordycepin currently on the market is isolated from cultivated *C. militaris*.

1.7.2 Structure of cordycepin

Cordycepin (3' deoxyadenosine) is an adenosine analogue that lacks the 3' hydroxyl group at the ribose sugar, which is important for 5'-3' elongation (Cunningham et al. 1950) as shown in the figure 1.11.

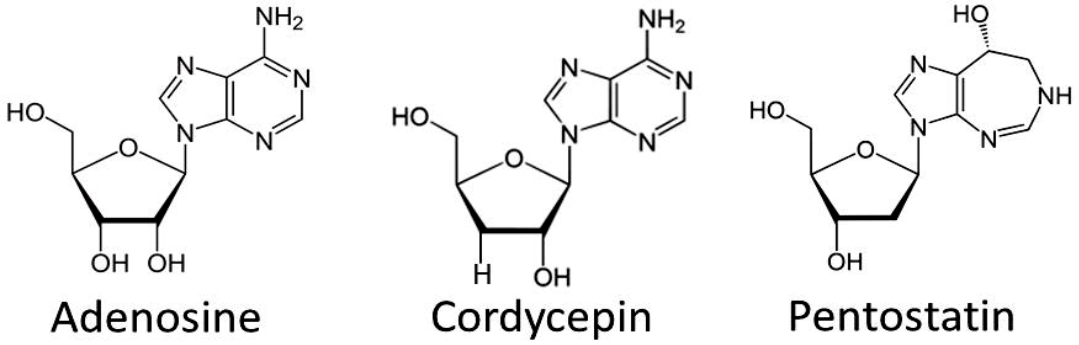


Figure 1.11: Chemical structures of adenosine, cordycepin and pentostatin.

Taken from: (Tsai et al. 2010).

1.7.3 Cordycepin as a cancer drug

Cordycepin has been proposed as an anticancer drug acting by many different mechanisms such as inhibition of translation, induction of nucleic acid methylation, apoptosis induction, anti-angiogenesis, antioxidation and regulation of signalling pathways (Ng and Wang 2005). Other diversetherapeutic properties of cordycepin have been reported, ranging from anti-herpes activity (de Julian-Ortiz et al. 1999), anti-fungal (Sugar and McCaffrey 1998), antibacterial (Ahn et al. 2000), anti-malarial (Trigg et al. 1971), anti-viral (Panicali and Nair 1978), anti-atherosclerosis (Won et al. 2009) to anti-parasitic (Rottenberg et al. 2005) activities.

1.7.3.1 Role of cordycepin in cancer cell survival and proliferation

Anticancer activity of cordycepin through the induction of apoptosis has been reported in many papers (Buenz et al. 2005, Wehbe-Janek et al. 2007, Wu et al. 2007, Chang et al. 2008, Chen et al. 2008, Thomadaki et al. 2008, Kitamura et al. 2011) in many typer of cancer, such as leukemia (Wehbe-Janek et al. 2007), oral cancer cells (Wu et al. 2007), bladder (Lee et al. 2009), breast cancer cells

(Thomadaki et al. 2005), HELA cells (He et al. 2009), mouse leydig tumor cells (Pan et al. 2011), human gallbladder (Wang et al. 2014) and lung cancerous cells (Nakamura et al. 2005, Nakamura et al. 2006). Cordycepin was reported to induce caspase-dependent apoptosis by activating reactive oxygen species (Thomadaki et al. 2008, Jeong et al. 2011) and caspase-independent apoptotic pathway by inhibiting RNA synthesis (Chen et al. 2010). Caspases are classed into upstream initiator caspases (caspases 2, 8, 9, 10) and effector caspases (caspases 3, 6, 7) which play crucial roles in inflammation and apoptosis (Earnshaw et al. 1999). Cordycepin has been reported in several papers to induce apoptosis by activating caspases (Park et al. 2005, Lee et al. 2006, Jin et al. 2008, Thomadaki et al. 2008, Park et al. 2009, Chen et al. 2010, He et al. 2010, Choi et al. 2011, Jen et al. 2011, Jeong et al. 2011). Activation of caspases leads to the disruption of crucial intracellular functional and structural proteins which results in apoptosis (He et al. 2010).

Deregulation of programmed cell death has been involved in increased cancer cell survival. Apoptosis is regulated by many intracellular signaling pathways, including the MAPK pathways. ERK (extracellular signal regulated protein kinase), c-jun N-terminal kinase (JNK) and p38 MAPK pathway are among the three MAPK pathways kinases (Liu and Lin 2005). Cordycepin has been reported to induce apoptosis by increasing protein expression of Bcl-2 pro-apoptotic members (Bax, Bim, Bid and Puma) and activating MAPK pathways kinases (JNK and p38) (He et al. 2010). Several papers indicate that cordycepin inhibits cell proliferation (Nakamura et al. 2005, Chang et al. 2008, Shi et al. 2008, Wang et al. 2014) including inhibiting the growth of HCT116 colon cancer cells by activating p21WAF1 expression (Lee et al. 2010) and in B16 (Mouse melanoma cell line) by inhibiting cyclin D1 (Yoshikawa et al. 2008). In addition, cordycepin has been reported to reduce protein synthesis by inducting the phosphorylation of eIF2α and dephosphorylation of 4EBP (Wong et al. 2010, Kitamura et al. 2011). Many of these studies claim to demonstrate the mechanism of action of cordycepin. However, a mechanism of action that demonstrates that the drug cordycepin directly affects the targeted process has not been identified in any of these cases.

As described previously, therapeutic agents have been selectively designed to target ER positive breast cancers, however these agents (ER modulators) are ineffective to ER negative breast cancers. In this regard, cordycepin has been investigated in treatment of breast cancer and found to be very effective in inducing apoptotic and autophagic cell death in MDA-MB-231 (basal like) cell and MCF-7 (ER positive) cells respectively. Thus cordycepin represses the in vitro growth of breast cancer cells irrespective to the presence of the ER. The exact mechanism of autophagic and apoptotic inducing cell death by cordycepin in breast cancer cells needs to be explored, but it may result from the inhibition of mTOR signalling that has been observed in other cell types (Wong et al. 2010). Cordycepin has been proposed as a potentially safe therapeutic agent for breast cancer treatment (Choi et al. 2011). The inhibition of cell proliferation by cordycepin in MCF-7 cells has also been reported by others (Ko et al. 2013).

1.7.3.2 The effect of cordycepin on DNA damage repair

Cordycepin has been reported to inhibit PARP (poly ADP-ribose polymerase) activity in BRCA1 mutated breast cancer MCF-7 cells. PARP is involved in repairing DNA single strand breaks (Kim et al. 2011). However, the inhibitor of PARP appears to be unmodified cordycepin, rather than cordycepin triphosphate, which is the predominant intracellular metabolite of cordycepin in MCF-7 cells (Utami, Khurshid, De Moor & Barrett. unpublished observation).

Double strand breaks can be repaired by protein complexes containing BRCA1 and BRCA2 (Jackson and Bartek 2009). In breast cancer with defective BRCA1 or BRCA2, PARP inhibition is considered as an effective mechanism to disable the DNA repair process (Weil and Chen 2011). Induction of the DNA double strand repair pathway was observed upon cordycepin treatment in breast cancer cells indicating the anti cancer effects of cordycepin may be through similar pathways as chemotherapy (Lee et al. 2012).

1.7.3.3 Cordycepin activates AMPK and inhibits PI3K/AKT and MAPK signaling pathways

Cordycepin reduces AKT kinase and 4E-BP phosphorylation by inhibiting the activities of the mTORC1 and mTORC2 complexes in PI3K/AKT/mTOR signaling pathway, while cordycepin activates AMPK by an unknown mechanism that in turn inhibits mTORC2 activity leading to a double block of the mTOR signalling. Thus reduces total proten synthesis. Activation of AMPK appears to be upstream of downregulation of the mTOR pathway. Inactivation of AKT (Guo et al. 2010, Wong et al. 2010, Jeong et al. 2012, Takahashi et al. 2012, Wu et al. 2014, Zhang et al. 2014) by cordycepin has also been reported. In one case, activation of AMPK by cordycepin also leads to reduction in ERK phosphorylation. This is thought to be through downregulation of BRAF, mediated by activated AMPK, thus suppressing the MAPK signaling cascade (Shen et al. 2013).

1.7.4 Molecular functions of cordycepin

Although there is some evidence for a direct interaction of cordycepin or its active metabolite cordycepin triphosphate with AMPK or PARP1 (Kim et al. 2011, Wu et al. 2014), the best characterised direct effect of cordycepin is its termination of polyadenylation through chain termination and arrest of the cleavage complex. Cordycepin reduces mRNA synthesis and acts as a chain terminator for mRNA polyadenylation through poly(A) polymerases, without affecting pre-mRNA levels (Shigeura and Gordon 1965, Penman et al. 1970, Horowitz et al. 1976, Muller et al. 1977, Rose et al. 1977, Glazer et al. 1978, Chen et al. 2010, Cheng et al. 2011). Cordycepin is definitely not a general transcription inhibitor (Kondrashov et al; 2012).

Cordycepin triphosphate has been widely used in *in vitro* polyadenylation reactions to elucidate the process of polyadenylation that is described in section 1.3. After chain termination, cordycepin arrests a complex of polyadenylation factors called the cleavage complex on the mRNA (Ryner and Manley 1987, Zarkower and Wickens 1987, Zarkower and Wickens 1987), which contains factors that are absent in other polyadenylation complexes (Shi et al. 2009), such as mRNA export factors. Normally, polyadenylation factors are released and the proto-oncoprotein

nucleophosmin is deposited on the mRNA before it is exported (Qu et al. 2009, Sagawa et al. 2011). In contrast, treatment of cells with cordycepin prevents the deposition of nucleophosmin on newly made mRNAs (Sagawa et al. 2011) as well as inhibiting polyadenylation (Kondrashov et al; 2012). Cordycepin specifically affects the expression of several inflammatory mRNAs in airway smooth muscle by a post-transcriptional route and their accumulation in the cytoplasm is prevented (Kondrashov et al; 2012). If affects of cordycepin are mediated through the inhibition of polyadenylation, then inhibitors of cordycepin import and phosphorylation should abrogate them. In addition, early effects of cordycepin can be predicted to be post-transcriptional. Both of these predictions have been observed (Kondrashov et al; 2012). In addition, transcription termination defects detected in cordycepin treated cells (Kondrashov et al; 2012), could account for the observation of DNA damage through the formation of R-loops (Hill et al. 2014, Skourti-Stathaki and Proudfoot 2014). These are hybrids that can form between transcribed RNA and DNA which can trigger a DNA damage response.

1.7.5 Effect of cordycepin on adenosine receptors

Adenosine and its receptors form a signaling pathway involved in the regulation of various tissue related functions. Low levels of adenosine are present in normal or unstressed cells compared to stressed or inflammatory states. In addition, genetic alterations which arise during cancer development enhance the amount of adenosine in the cell (Antonioli et al. 2014). In the extracellular environment high levels of adenosine stimulate adenosine receptors resulting in the activation of cell signaling cascades and the inductuon of anti-inflammatory and immune responses. In addition activation of adenosine receptors can lead to increased proliferation, apoptosis and metastasis of cancer cells (Antonioli et al. 2013, Virtanen et al. 2014). Four different G-protein coupled membrane receptors are responsible for action of extracellular adenosine. Activation either increases (A_{2A} and A_{2B}) or reduces (A_1 and A_3) intracellular levels of cAMP (Fredholm et al. 2001, Sorrentino et al. 2013).

The strength of adenosine signalling relies on the amount of adenosine. Adenosine receptors interact with MAPK kinase pathway and adenosine also acst without its receptor through AMPK and adenosine kinase (Antonioli et al. 2013). Various papers

have been reported that stimulating an adenosine receptor, mainly A_3, is involved in suppressing cell proliferation in various cancers. This is thought to be through G_0-G_1 cell cycle arrest by inhibiting the activity of cyclin D1, cyclin E1 and CDK4, which decreases the levels of AKT and downregulates the phosphorylation of ERK (Fishman et al. 2000, Fishman et al. 2002, Merighi et al. 2002, Madi et al. 2003, Morello et al. 2008, Aghaei et al. 2011). However, the role of other adenosine receptors in inhibiting cell proliferation needs further investigation (Antonioli et al. 2013). Adenosine receptors can induce apoptosis in cancer cells by increasing the levels of CD95 (death receptor), TRAILR2 (tumour necrosis factor-related apoptosis-inducing ligand receptor 2), FADD (FAS-associated death domain protein). This leads to activation of caspases 8 and 9 followed by caspase 3 and decreases the levels of anti-apoptotic protein such as BCL-2, in addition to the decrease of phosphorylation of AKT. It also increases the levels of BAX, BAD modulators of apoptosis (Kim et al. 2002, Wen and Knowles 2003, Lee et al. 2005, Bar-Yehuda et al. 2008, Yasuda et al. 2009, Yang et al. 2010, Cohen et al. 2011, Aghaei et al. 2012, Mlejnek et al. 2012, Tamura et al. 2012, Antonioli et al. 2013, Chen et al. 2014).

Cordycepin also has been proposed to prevent tumor growth formation through activating adenosine Gi protein-coupled A_3 receptors (Fishman et al. 2002, Nakamura et al. 2006, Yoshikawa et al. 2008, Kitamura et al. 2011). It was reported previously that cordycepin acts through adenosine receptors (A_1, A_{2A} and A_3) in stimulating steroidogenesis in mouse leydig cells, while it may act through A_1, A_{2A}, A_{2B} and A_3 receptors in MA-10 cells (Leu et al. 2011, Pan et al. 2011, Pao et al. 2012). Higher levels of mRNA expression of A_3 adenosine receptors were reported in human breast and colon cancerous cells compared to non cancerous cells (Yoshikawa et al. 2008). The mechanism of action of cordycepin through activation of adenosine receptors predicts that adenosine and cordycepin should have similar activities and that inhibition of cordycepin import or phosphorylation should not prevent the activity of cordycepin. These predictions do not hold true in many cell types (Kondrashov et al; 2012; Wong et al; 2010).

1.7.6 Pharmacokinetics of adenosine and cordycepin

Adenosine deaminase is a catabolic enzyme that plays an important role in purine metabolism. There are two isoenezymes ADA1 and ADA2. ADA1 is involved in the deamination process of adenosine and 2'deoxyadenosine (Kayhan et al. 2008). Normal endogenous levels of adenosine in plasma are 0.1-1µM. Adenosine and cordycepin are irreversibly deaminated by adenosine deaminase into hypoxanthinosine as shown in the figure 1.12 and to an inactive form 3'deoxyhypoxanthinosine in the plasma respectively. The half life of adenosine in plasma is found to be 0.6-1.5 seconds (Tsai et al; 2010).

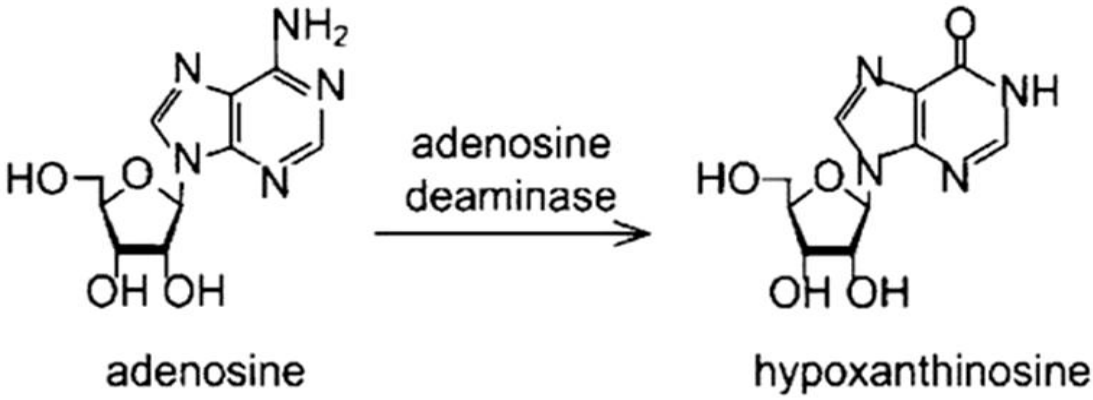


Figure 1.12: Deamination of adenosine into hypoxanthinosine.

Taken from: (Tsai et al. 2010).

Nucleoside permeases are responsible for transporting adenosine into the cell where it is phosphorylated to form ATP by adenosine kinase, which also phosphorylates cordycepin into 3'deoxyadenosine phosphate derivatives. Due to the instability of cordycepin it is usually administered in combination with another purine analog, 2'- deoxycoformycin (dCF), also known as pentostatin. However pentostatin has considerable adverse effects, including severe immune suppression. In contrast, cordycepin on its own has very few side effects. Co-administration of an ADA inhibitor eg. pentostatin increases cordycepin efficacy both *in vivo* and vitro, but it also results in gastrointestinal and bone marrow toxicity. (Rodman et al. 1997). Combination of pentostatin with cordycepin cures *Trypanosoma brucei* infection in mice. The parasitic infection was found to be eliminated from the blood

and also from the brain parenchyma (Rottenberg et al. 2005, Vodnala et al. 2008). It was also reported that the combination of cordycepin and pentostatin effectively cures mice in vivo and vitro infected with *Trypanosome evansi* (Dalla Rosa et al. 2013).

Pentostatin is an adenosine deaminase (ADA) inhibitor which inhibits the deamination of both adenosine and 2'-deoxyadenosine to form inosine and 2'deoxy-inosine respectively. In addition, it has been reported to block DNA synthesis during the "S" phase of the cell cycle through inhibition of ribonucleotide reductase and through incorporation into DNA as a false purine base. Additional cytotoxic effects have been attributed to its incorporation into RNA (Tsai et al. 2010).

The exact mechanism of action of pentostatin is still unknown. However it is currently used to treat hairy cell leukemia and found to inhibit DNA repair, although toxicity issues have been reported (Airhart et al. 1996, Margolis and Grever 2000, Lauria and Forconi 2009, Robak and Robak 2012). Therefore due to a high rate of clearance and short half lives of adenosine and cordycepin, addition of pentostatin will reduce the degradation rate in plasma. Toxicity of cordycepin with pentostatin for acute lymphocytic leukemia was found to be tolerable in humans and further clinical trials are being coordinated by Oncovista (Foss 2000) (website reference 1).

1.7.7 Mode of action of C-8 substituted adenosine analogues

Modifications of cellular nucleosides are based on structural or conformational changes. They can be placed in the ribose sugar or the base such as 8-amino adenosine, 8-chloro adenosine etc as shown in figure 1.13. Such nucleoside homologs have been widely tested as a chemotherapeutic agent. In general, purine bases are studied more extensively for cancer therapy (Chen and Sheppard 2004). Adenosine analogues can decrease the endogenous levels of ATP by accumulating as triphoshate in the cell (Anders et al. 2010). The application of these C-8 substituted adenosine analogues as an antineoplastic drug has been reported in many studies. The 8-amino adenosine and 8-chloro adenosine may induce apoptosis due to a decline in cellular ATP levels (Halgren et al. 1998, Gandhi et al.

2001, Chen et al. 2010). 8-chloro adenosine acts as a transcription inhibitor resulting in low levels of total cellular RNA due to an imbalance between RNA biosynthesis and the rate of its turnover, by slowing down the process of transcription and increasing mRNA degradation (Stellrecht et al. 2014). Addition of C-8 adenosine analogue disrupts chain extension during RNA transcription but also causes termination of the poly(A) tail, which results in inhibition of RNA transport or processing (Chen and Sheppard 2004). Adenosine analogues may exert their effects by disrupting other ATP dependent RNA polymerases, for example poly(A) polymerases that have a structural similarity to ATP (Chen and Sheppard 2004).

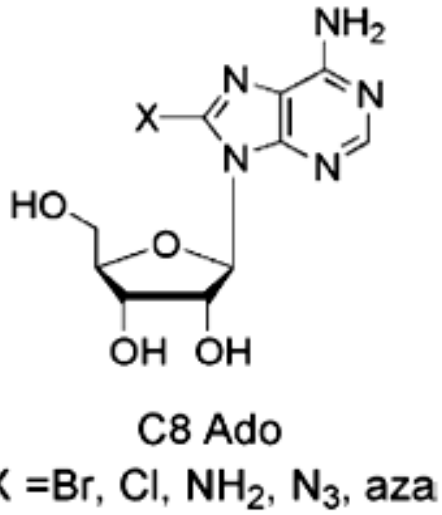


Figure 1.13: Chemical structure of C-8 substituted adenosine analogue.

Taken from: (Chen et al. 2010)

C-8 substituted adenosine analogues cause conformational changes by changing the sugar equilibrium to the C-2' endo form like DNA over C-3' endo RNA like conformation. So far, among C-8 substituted adenosine analogues, 8-amino ado and 8-azido ado have been reported to act similarly to cordycepin as chain terminators. They are thought to inhibit RNA polyadenylation by incorporating into nascent poly(A) tails. Differences in the polyadenylation inhibitory activities were also observed *in vitro* among these C-8 substituted adenosine analogues, depending upon the substitution. Nitrogenous substitution at C-8 position like 8-amino ado (adenosine), 8-aza ado and 8-azido ado resulted in a moderate inhibition of

polyadenylation compared to the halogen substitution at C-8 position. This is similar to 8-chloro ado and 8-bromo ado which markedly inhibited polyadenylation as they act as a weak substrate for PAP, decreasing the efficiency of transfer of AMP to the substrate from the enzyme. It was also proposed that the induction of glycosidic bond conformational changes in sugar play a role in these inhibitory effects. The main difference between preferred sugar conformations in ribonucleotides and deoxy ribonucleotides are due to the C-3'- endo for ribonucleotides and C-2'-endo for deoxy ribonucleotides (Chen and Sheppard 2004).

1.7.7.1 8-Chloro adenosine analogue

The cytotoxic effects of 8-chloro adenosine have been reported in several papers (Gandhi et al. 2002, Balakrishnan et al. 2005, Dennison et al. 2009) by suppressing transcription (Stellrecht et al. 2007) and PAPs (Chen and Sheppard 2004, Chen et al. 2010) through incorporation into the mRNA (Stellrecht et al. 2003). 8-chloro adenosine has been reported to induce apoptosis in breast cancer by inhibiting the levels of cyclin E (Stellrecht et al. 2010) and like cordycepin by reducing the levels of cellular ATP. This in turns caused AMP and upstream kinases to activate AMPK in addition to downregulating levels of P-4EBP by inhibiting the PI3K/mTOR signaling cascade (Stellrecht et al. 2014). Currently 8-chloro adenosine is in phase I clinical trials (Pillai et al. 2012, Polotskaia et al. 2012). 8-chloro adenosine is the only purine analogue reported so far to be effective in multiple myeloma cell lines (Krett et al. 1997, Halgren et al. 1998, Gandhi et al. 2001, Stellrecht et al. 2003) at inducing apoptosis by interfering with RNA synthesis, without exerting any effect on DNA synthesis. Like cordycepin, 8-chloroadenosine needs to be phosphorylated to exert its effects on cellular targets (Gandhi et al. 2001, Stellrecht et al. 2003). Unlike cladribine which gets phosphorylated by deoxycytidine kinase, 8-Cl-ado requires adenosine kinase to be metabolized intracellularly to 8-Cl-cAMP (Stellrecht et al. 2003). In cells, inhibition of mRNA synthesis results from chain termination upon significant incorporation of 8-chloro adenosine by Pol II into the transcript, which might be due to the impaired base pairing between adenine and thymine. This results in a lack of hydrogen bonding between the DNA template and the newly formed transcript. In addition, Pol II may be more sensitive to the decreased levels

of ATP (Stellrecht et al. 2003). Degradation of 8 chloro adenosine also involves deamination by adenosine deaminases to 8-chloro-inosine monophosphate (Tsai et al. 2010).

1.8 Aims of the project

Cordycepin could interfere with many normal cell metabolic functions, as adenosine is used ubiquitously in the body and at least four direct targets have been proposed in the literature (adenosine receptors, AMPK, PARP1 and polyadenylation). The aim of my project is to investigate the mechanism of action of cordycepin and its potential as an anticancer drug by studying its effect on protein synthesis, cell proliferation and gene expression. I will be comparing the effects of cordycepin with those of other adenosine analogues and the knowckdown of poly(A) polymerase. A microarray study will be used to identify mRNAs that are affected by cordycepin treatment.

2 Materials and Methods:

2.1 General reagents

2.1.1 Reagent and equipment suppliers

New England Biolabs (NEB) 75 - 77 Knowl Piece, Wilbury Way, Hitchin, Herts. SG4 0TY

Fisher-Scientific Bishop Meadow Road, Loughborough, Leicestershire. LE11 5RG

GE Healthcare (Amersham) GE Healthcare Life Sciences, Amersham Place, Little Chalfont, Buckinghamshire. HP7 9NA

Invitrogen Ltd. 3 Fountain Drive, Inchinnan Business Park, Paisley, UK. PA4 9RF

Sigma-Aldrich Company Ltd. The Old Brickyard, New Road, Gillingham, Dorset. SP8 4XT

Roche Products Limited. 6 Falcon Way, Shire Park, Welwyn Garden City. AL7 1TW

Promega, UK. Delta House, Southampton Science Park, Southampton. SO16 7NS

2.1.2 Chemicals

Cordycepin (3'-Deoxyadenosine), 8- Bromoadenosine, DRB (5, 6-Dichloro-1-beta-D-ribofuranosylbenzimidazole), Cladribine (2-Chloro-2'-deoxyadenosine) and 5'-Deoxy-5'-(methylthio) adenosine were purchased from Sigma. They were dissolved at a thousand fold concentration in DMSO to obtain the indicated final concentrations. Metformin (1, 1-Dimethylbiguanide hydrochloride) was also purchased from Sigma and dissolved upto 50mM in DMSO. 8- Azidoadenosine (8-N3-Ado) was purchased from Biolog. 8-Chloroadenosine and 8-aminoadenosine were purchased from Carbosynth and Santa Cruz respectively. Pentostatin was purchased from Tocris. Se13 and Se15 confidential compounds were gifted by Dr Shudong Wang, Medicinal chemistry division, School of Pharmacy, University of Nottingham.

2.2 Tissue culture techniques

2.2.1 Solutions and reagents

Phosphate buffered saline (PBS): 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4. (OXOID)

Dulbecco's Modified Eagles Medium (DMEM) (PAA) with 4.5 g/L of glucose and 4 mM glutamine

McCoy's 5a Medium (Sigma)

Feotal Bovine Serum (FBS) and Foetal calf serum (FCS) heat-inactivated (PAA)

Penicillin-Streptomycin, 10,000 unit's penicillin and 10 mg streptomycin/ml, sterile-filtered (Sigma-Aldrich)

2.2.2 Cell Lines

Table 5: Decription of Cell lines

Cell line	Origins	Description
HELA	Human cervical epithelial carcinoma	Adherent cell line maintained in DMEM supplemented with 10% FBS and 4mM L-glutamine
MCF-7	Human breast epithelia adenocarcinoma	Adherent cell line maintained in DMEM supplemented with 10 % FBS, Penicillin-Streptomycin 10ml/L and 4 mM L-glutamine
NIH3T3	Mouse embryonic fibroblast cells	Adherent cell line maintained in DMEM supplemented with 10 % FCS and 2 mM L-glutamine
HT29	Human colorectal adenocarcinoma cells	Adherent cell line maintained in McCoy's 5a Medium supplemented with 10 % FBS, and 4 mM L-glutamine
HCT116	Human colorectal adenocarcinoma cells	Adherent cell line maintained in McCoy's 5a Medium supplemented with 10 % FBS, and 4 mM L-glutamine
A549	Human lung adenocarcinoma cell line	Adherent cell line maintained in DMEM supplemented with 10 % FBS, and 4 mM L-glutamine

2.2.3 Culturing and Maintenance of cell lines

All cell lines used were purchased from the European Collection of Cell Cultures (ECACC). Cells were cultured in gamma sterilized plasticware (TPP c/o Helena

Biosciences). NIH3T3 (Mouse fibroblast cell line) were cultured in calf serum while the rest of the cell lines were cultured in FBS. McCoy's 5A Medium was used to culture HCT116 and HT29 (Human colon carcinoma).

For routine culture, cells grown to full confluence were washed with phosphate buffered saline (PBS) and treated with 1X Trypsin-0.5mM EDTA (PAA) for 5min at 37^{o}C. Once cells had detached, fresh medium was added to neutralise the trypsin and 20-40% seeded into a new flask and passaged every couple of days as required. Cells were maintained for no more than 20 passages. Where a specific seeding density was required, a haemocytometer was used to count cell density from a neutralised sample. For each experiment cells, were seeded at a required density one day before use or as indicated. Cells were washed with PBS, scraped and detached by trypsinization. All cells were cultured in gamma sterilised, tissue culture treated plasticware (TPP).

2.2.4 Isolation and quantification of total RNA from Tissue culture cells

Total RNA was isolated from tissue culture cells by using Total RNA Isolation kit (NucleoSpin® RNA II) by MACHEREY-NAGEL (Germany) according to the manufacturer's instructions by affinity purification. RNA was quantified by nanodrop and the purity was assessed by a ratio of absorbance at 260 nm and 280 nm (generally ~1.8 is accepted for RNA). Ratios lower than ~1.8 indicates the presence of contaminants such as protein or phenol that also absorbs strongly near 280nm.

2.3 Molecular Biology Techniques

2.3.1 Buffers and solutions

TE: 10 mM Tris-HCl, pH 7.5; 1 mM EDTA.

1 x TBE: 89 mM Tris base; 89 mM boric acid; 2.5 mM EDTA; pH 8.0.

RNA loading dye: 50 % (v/v) glycerol; 1 mM EDTA, pH 8.0; 0.25 % (w/v) bromophenol blue; 0.25 % (w/v) xylene cyanol.

UREA loading dye for 3' end Labeling: 8 M Urea, 10mM EDTA, bromphenol blue (0.025%) w/v and Xylene cyanol (0.025%) w/v.

2.3.2 Separation of Nuclear /Cytoplasmic fractions

Nuclear and cytoplasmic fractions were separated according to (Felton-Edkins et al. 2006), with minor changes. Cells were plated at a density of 2.5 million cells in two 15 cm plates the day before use for each treatment. Cells were treated for 2 hours with Cordycepin (50µM) at 37°C and washed and scraped in chilled PBS and cells pellet were collected by centrifugation at 2000 rpm for 5 minutes. The cell pellet for each treatment was lysed in 400µl of lysis buffer (Hepes 10mM pH 7.9, KCl 10mM, EDTA 0.1mM, EGTA 0.1mM, DTT 1mM, PMSF 0.5mM) (DTT and PMSF add freshly at time of use) followed by short incubation on ice for 15 minutes. The cells were vortexed and placed on ice for 3-5 minutes after the addition of 25 µl of 10% NP-40. Nuclei were collected by short spin at maximum speed for 40-45 seconds. For cytoplasmic fraction isolation, the supernatant was collected in a fresh tube and precipitated with 1 volume isopropanol for at least 30 minutes at -20° followed by centrifugation at 4°C for 20 minutes at maximum speed. The pellet was washed with 500 µl of 70% ethanol, then centrifuged at maximum speed at 4°C for 5 minutes. The washed pellet was resuspended in 350 µl of lysis buffer (Total RNA Isolation kit) for RNA Isolation.

For the nuclear fraction, the pellet (nuclei) was resuspended in lysis buffer (400µl), vortexed and placed on ice for 5 minutes, followed by centrifugation at maximum speed for 1-5 minutes. The nuclei pellet was lysed in a 350µl of lysis buffer (Total RNA Isolation kit) for RNA isolation.

Cytoplasmic and nuclear RNA were isolated from individual fractions using Total RNA Isolation kit as described previously according to manufacturer's instructions. RNA was quantifyied by nanodrop and purity was assessed as described previously and used for RT-qPCR.

2.3.3 Quantitative PCR

RNA was reverse transcribed using Superscript III (Invitrogen) and random hexamers (Invitrogen). Quantitative PCR was performed in triplicate on each sample using Go Taq qPCR Master Mix (Promega) according to the manufacturer's instructions by using ROX as a reference dye. Primers were used in final concentrations of 0.5µM and 0.25 µM for test and control mRNAs respectively in a volume of 1µl each set of forward and reverse primer in a final reaction volume of 20µl. Melting curves were observed for each reaction and RNA levels were expressed relative to GAPDH or 18S ribosomal mRNA levels. Untreated control values (no cordycepin or no SiRNA) were set at 1.

2.3.3.1 Human primer sequences for RT-qPCR

Table 6: Primers sequences for RT-qPCR:

Name	Sequence	mRNA	unspliced
PTGS2- F	TGAAACCCACTCCAAACACA	x	
PTGS2-R	GAGAAGGCTTCCCAGCTTTT	x	
ACTB-F	CCAACCGCGAGAAGATGA	x	
ACTB-R	CCAGAGGCGTACAGGGATAG	x	
CCL2-F	ACTGAAGCTCGCACTCTCGC	x	
CCL2-R	AGCCTCTGCACTGAGATCTTC	x	
CXCL1-F	GCAGACCCTGCAGGGAATTC	x	x
CXCL1-R	GCCACCAGTGAGCTTCCTCC	x	
CXCL1 intron R	AGGGAATCTCGTGAGGCAGG		x
IL8-F	CCAAGCTGGCCGTGGC	x	
IL8-R	GCTCTCTTCCATCAGAAAG	x	x
un-IL8-FW	GGACTTAGACTTTATGCCTGA		x
GAPDH-F	CATCGCTCAGACACCATGGG	x	
GAPDH-R	CGTTCTCAGCCTTGACGGTG	x	
RPL10A-F	TCTCTCGCGACACCCTGT	x	x
RPL10A-R	TTAGCCTCGTCACAGTGCTG	x	
RPL10A Rev2	AGAGAGGAGGGGGGTTAAG		x
PAPOLA -F	GGGATCACAACAAACACAACC	x	
PAPOLA -R	TGCAGTTCCTCTTCCTCTTCA	x	
PAPD4 -F	CCACCCTTCACTCCAAATCA	x	
PAPD4 -R	CGAAATAATGGGGAAGCTGA	x	
PAPOLG-F	GCGGACTTCAATCCAAAAGA	x	
PAPOLG-R	CACTCAGTGGCTTCTCCACA	x	
HIF1A -F	CCGCTGGAGACACAATCATA	x	
HIF1A -R	AGCGGTGGGTAATGGAGAC	x	
TWIST1 -F	CTGGACTCCAAGATGGCAAG	x	
TWIST1 -R	TCCATTTTCTCCTTCTCTGGAA	x	

PIK3CA -F	CCCCGAAATTCTACCCAAAT	x	
PIK3CA -R	GAACAGCAAAACCTCGAACC	x	
PIK3CB -F	TGGCCTCATTGAAGTTGTGA	x	
PIK3CB -R	GCCAGCACAGGACAGTGTAA	x	
PIK3CD -F	AAGATCGGCCACTTCCTTTT	x	
PIK3CD -R	TGAGAGCTCAGCTTGACGAA	x	
IRS1 -F	ACGTGCGCAAGGTGGGCTAC	x	
IRS1 -R	CGTTTGGGGGCGCTCGACTT	x	
IRS2 -F	CCACCCCGCCGCAACCTATC	x	
IRS2 -R	GGGGTTGTGGGCGAAGGACG	x	
AKT1 -F	AGAAGCAGGAGGAGGAGGAG	x	
AKT1 -R	TCTCCTTCACCAGGATCACC	x	
AKT2 -F	TCCTCCACGACTGAGGAGAT	x	
AKT2 -R	GCAGGATCTTCATGGCGTAG	x	
AKT3 -F	CAGTAGACTGGTGGGGCCTA	x	
AKT3 -R	ATCAAGAGCCCTGAAAGCAA	x	
TP53 -F	GTGGAAGGAAATTTGCGTGT	x	
TP53 -R	CCAGTGTGATGATGGTGAGG	x	
RN18S -F	GAAACTGCGAATGGCTCATTAA	x	
RN18S -R	GAATTACCACAGTTATCCAAGTAGGA	x	
WEE1 (F)	GGGGAATTGATTCCAGCTCT	x	
WEE1 (R)	CACTGGCTTCCATGTCTTCA	x	
MED9 (F)	CCCTCAAAAGCAAGTTCCAG	x	
MED9 (R)	GGGATTTCGAACATGCAGAG	x	
LIF-F	GCCACCCATGTCACAACAACC	x	
LIF-R	GCTTGGCCTTCTCCGTGC	x	
c-JUN (F)	ACA GAG CAT GAC CCT GAA CC	x	
c-JUN (R)	CCG TTG CTG GAC TGG ATT AT	x	
FBXW7 (F)	TTGGACCATGGTTCTGAGGT	x	
FBXW7 (R)	TGTAGGTGGCTGGACAGATG	x	
PLK2 (F)	AAAGGTGTTGACAGAGCCAGA	x	
PLK2 (R)	AGACCGAAGTCCCCAACTTT	x	
ZFP36 -F	ACTTCAGCGCTCCCACTCT	x	
ZFP36 -R	GACTCAGTCCCTCCATGGTC	x	
SNAI1 (F)	CTCTAGGCCCTGGCTGCTAC	x	
SNAI1 (R)	CTTGTGGAGCAGGGACATTC	x	
ZFP36L1 (F)	GTCTGCCACCATCTTCGACT	x	
ZFP36L1 (R)	GCTGGTTCTGGTGGAACTTG	x	
NFKBIA (F)	CTACACCTTGCCTGTGAGCA	x	
NFKBIA (R)	TCCTGAGCATTGACATCAGC	x	
JUNB (F)	AAGGGACACGCCTTCTGAAC	x	
JUNB (R)	AAACGTCGAGGTGGAAGGAC	x	
EGR1 (F)	CAGCACCTTCAACCCTCAG	x	
EGR1 (R)	AGCGGCCAGTATAGGTGATG	x	
CSTF1 (F)	GATCCTGGCTTCTGGTTCAA	x	
CSTF1 (R)	AGGCGAAGAGTAGGATGCTG	x	
CNOT4 (F)	TCCAAGTGCCAGTGCTTATG	x	

CNOT4 (R)	TGCAGTCAGGTTTTGGACAC	x	
BTG1 (F)	CCATGCATCCCTTCTACACC	x	
BTG1 (R)	GCTTTTCTGGGAACCAGTGA	x	
IL6 -F	AAAGAGGCACTGGCAGAAAA	x	
IL6 -R	AGCTCTGGCTTGTTCCTCAC	x	
NEK6 (F)	ATCCCAACACGCTGTCTTTT	x	
NEK6 (R)	GCCGATCTCCTTGACACAGT	x	
MCL1 (F)	AGAAAGCTGCATCGAACCAT	x	
MCL1 (R)	CCAGCTCCTACTCCAGCAAC	x	
MED13L (F)	CACTTGGCAGCAGTTCCATA	x	
MED13L (R)	CAACAAGAGCCTCTCCCAAA	x	
MED26 (F)	CCAGCCTGGAGAAATACCCTA	x	
MED26 (R)	GGCTCGATGAGCTTCTGC	x	
ATF4 (F)	TCAAACCTCATGGGTTCTCC	x	
ATF4 (R)	GTGTCATCCAACGTGGTCAG	x	
TFPI (F)	GCCAGATTTCTGCTTTTTGG	x	
TFPI (R)	TGCGGAGTCAGGGAGTTATT	x	
DCP1A (F)	AAGACAGCAGCAGCAAGAGT	x	
DCP1A (R)	CGGGATGGCACTGGATAACA	x	
CD68 (F)	ACTGAACCCCAACAAAACCA	x	
CD68 (R)	TTGTACTCCACCGCCATGTA	x	
SUN1 (F)	AACAACAAAACAGCGCAGAA	x	
SUN1 (R)	TCCCTGAATGGCAGCTTTAT	x	
CTSL1 (F)	ACAGTGGACCAAGTGGAAGG	x	
CTSL1 (R)	TGGGCTTACGGTTTTGAAAG	x	
CPE (F)	TCTTGGCCCAGTACCTATGC	x	
CPE (R)	GTCTGGAAAGTTCCGGTTCA	x	
GNAI3 (F)	GCACCATTGTGAAACAGATGA	x	
GNAI3 (R)	CAGCTTCCCCAAAGTCAATC	x	
CATSPER2 (F)	ATCCTTTGGTTGTTGCTTGG	x	
CATSPER2 (R)	TGACGTCAGTGCTTCATGTG	x	

2.3.4 siRNA Transfections

Poly(A) polymerase mRNAs were knocked down by using the Smartpool siRNA (5 nM) against indicated PAPs (Dharmacon) (in case of combination PAP knockdowns the concentration of each siPAP was adjusted to 5 nM), by using INTERFERin (Polyplus) in tissue culture cells according to the manufacturer's instructions. Cells were seeded at a density of 0.075 million cells per well in a 12 well plate a day before first transfection followed by a medium changed at 24 hours and a second transfection was performed. Forty eight hours later, the cells were harvested and processed for total RNA isolation as described.

2.3.5 Protein synthesis assay by ^{35}S Methionine Incorporation

To determine the protein synthesis rate in tissue culture cells, 50,000 cells were seeded one day before use in a 24-well plate in 1ml media in 4-6 replicates. The next day, cells were incubated with the required concentration of different adenosine analogues and incubated for 2 hours.

The medium was then removed and the cells were washed twice with PBS and 5-15 µCi/ml Tran^{35}S-methionine label (Perkin Elmer) was added to pre-warmed cysteine and methionine-free DMEM, and returned to the incubator. After 10-15 minutes the cells were placed on ice, washed with PBS containing 1% (w/v) L-Methionine, and lysed with 50µl 1X Passive lysis buffer (Promega) per well. Incorporation was measured by TCA precipitation on Whatman 3MM paper.

10µl of lysate were used to be spotted on to Whatman 3MM filter paper and 10 µl were used for total protein quantification using Bradford reagent (Bio-Rad) in a 96 well micro plate, according to the manufacturer's instructions.

Filters were incubated for a few minutes in 10% TCA with a pinch of methionine and cysteine at room temperature and then in 5% TCA for few a minutes. The filters were then washed twice with 98% industrial methylated spirits, and left to air dry. Radiolabeled protein was liberated using Ecoscint scintillation fluid (National Diagnostics) and quantified in a Wallac 1409 scintillation counter (Pegasus Scientific). Incorporation was corrected for protein content using the Bradford data. All incorporations were plotted as a percentage of the incorporation in the untreated control cells.

2.3.6 In cell-enzyme linked immunosorbent assay (ELISA) for phospho 4E-BP as a cordycepin bioassay

Cells were seeded at a density of 20,000 cells per well in 4-5 wells for each treatment in 96 well plates in 100 µl medium the day before use. One set of replicates were the no primary antibody control, which were used as background. After the indicated treatments for 2 hours, cells were washed twice with PBS followed by incubation with 4% paraformaldehyde for 15 minutes. Cells were

washed again with PBS and incubated in 100µl of 0.1% Triton for 15 minutes. Cells were washed with PBS and incubated in 3% BSA for 30 minutes.

In empty wells 40µl of anti phospho 4EBP is added at a concentration of 1:500 in 3% BSA, except for the background wells. After an overnight incubation at 4°C, cells were washed again with PBS and probed for 2 hours at RT with 40µl of anti-rabbit IgG-HRP in 1:1000 in 3% BSA followed by PBS washing. Cells were analyzed by using 3, 3', 5, 5'-Tetramethylbenzidine (TMB) Liquid Substrate System substrate for horseradish peroxidise from sigma. The reaction was stopped by 1N sulphuric acid, forming a yellow reaction product that was read by plate reader Biotek at 450 nm and background values were subtracted from the readings. All values were plotted as a percentage of the average for the untreated control cells.

2.3.7 3'-End labeling of mRNA with ^{32}PpCp (Cytidine 3', 5'-bisphosphate (5'-^{32}P))

RNA was prepared for 3' end labeling as described in detail by (Minvielle-Sebastia et al. 1991) with minor changes. 250ng of RNA was incubated in 7.5µl of reaction volume containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 8.1), 5uM ATP, 10 mM $MgCl_2$, 3.3 mM dithiothreitol, 10% dimethyl sulfoxide, 300ug/ml of bovine serum albumin (BSA), 12.5 uCi/µl of Cytidine 3',5'-[5'-^{32}P]biphosphate (^{32}P-pCp; Perkin Elmer), and 3 U of T4 RNA ligase (New England BioLabs) overnight at 4°C.

Next day the labelled RNAs were treated with RNase A (Sigma) and RNase T1 (Fermentas) for 2 h at 37°C, to cleave everything except the poly (A) tail in a 12.5µl reaction mixture that contained the 7.5ul of initial reaction mixture, 0.8mg/ml of yeast tRNA (Ambion), 16 mM Tris (pH 7.5), 0.48 M NaCl, water and RNase cocktail (20 Units/µl of RNase T1, 50ng of RNase A in 50mM Tris (pH 7.5), 50% glycerol).

Following this treatment, labelled RNA was subjected to Proteinase K treatment in 7.5µl of initial reaction mixture containing proteinase K (2 mg/ml; NEB), 130 mM EDTA and 2.5% SDS for 30 min to 1 hour at 37 °C. Samples can be stored at -20 ° at this stage if required.

Labelled samples were processed for phenol-chloroform-isoamyl alcohol (25:24:1) extraction by adding equal volume of water in the sample. RNA was precipitated in absolute ethanol with 3M sodium acetate and 1.25µg glycogen as a carrier at -20°C. The pellet is resuspended in 8M urea gel loading buffer.

The samples were denatured at 65 °C for 5-10 min and run on a 15% polyacrylamide-urea gel (acrylamide/bisacrylamide, 30:1; 7 M urea) in 0.5X TBE at 20watts for 4 hours. The gels were autoradiographed overnight at -80°C and scanned the next day on phosphoimager STORM 825. Labelled RNA ladder was used as a marker where each band represents different sizes of poly(A) tail lengths in bases.

2.3.8 Proliferation Assay

Cells were counted using trypan blue staining (Sigma). The cells which exclude the dye are viable with intact cell membrane and retain as white. Cells were seeded in a 6 well plate at a density of 0.15 million cells per well. After 24 hours, cells were processed for siRNA transfections (as described previously) or treated with indicated compounds for 72 hours. The cells were washed with PBS and collected by trypsinization and counted under the microscope using a haemocytometer. Each experiment was performed in four to five replicates. Data are represented as a relative number of cells by normalizing to its original seeding density.

2.3.9 Immunohistochemistry of MCF-7 cells

In order to observe focal adhesion changes by immunohistochemistry, MCF-7 cells were seeded in a 24 well plate at a density to 15,000 cells per well on round glass coverslips of 13 mm, thickness 1, borosilicate glass (VWR 631-0149). Cells were treated after 48 hours with indicated concentration of cordycepin for 3 hours.

After treatment, cells are washed carefully with PBS and fixed with 4% paraformaldehyde. After a couple of washes with PBS, cells were incubated in 0.1% Triton X100 in PBS for 10 minutes then washed again with PBS and followed by an hour incubation at room temperature with 3% bovine serum albumin in PBS. Cells were incubated with primary antibody (Vinculin from sigma) in 0.25 ml 3% BSA in PBS, incubated O/N at 4°C at 1:200. PBS was used to wash the cells followed by 1 hr

incubation at room temperature in secondary antibody of anti-mouse-coupled to Alexa 546 (Molecular Probes) at 1:200 in 3% bovine serum albumin in PBS.

After a couple of washes with PBS, cover slips were mounted on slides with the help of mounting fluid. Imaging was performed on a Zeiss LSM510 Metaconfocal microscope at 63X magnification, and the images were processed using the manufacturer's software.

2.4 Microarray profiling

Cells were seeded at a density of 2.3 million cells per 15 cm plate and incubated for 24 hours. Cells were treated for 2 hours with DMSO and Cordycepin (50µM). Cells were washed gently with PBS to remove excess medium after the treatment and cells were scraped in 5ml of ice cold PBS followed by centrifugation. Collected cells were processed for total RNA isolation as described previously. Experiments were done in a four independent biological replicates.

Microarray experiments were performed in quadruplicate in collaboration with Prof. Anne Willis**,** MRC Toxicology Unit University of Leicester using Agilent GE 60k human arrays. Each sample was hybridized in randomly assigned locations onto an individual microarray consisting of 8 on each slide.

The samples were labelled using Agilent's one-colour Low Input Quick Amp Labelling Kit i.e.the dye used was cy-3. The slides were scanned using an Agilent Microarray Scanner, model number G2505C, scan control version A.8.4.1. The data was extracted using the Agilent Feature Extraction software version 10.7.3.1. The platform used was: Human GE 8x60k v.2 microarrays. The R software package was used to analyse the data.

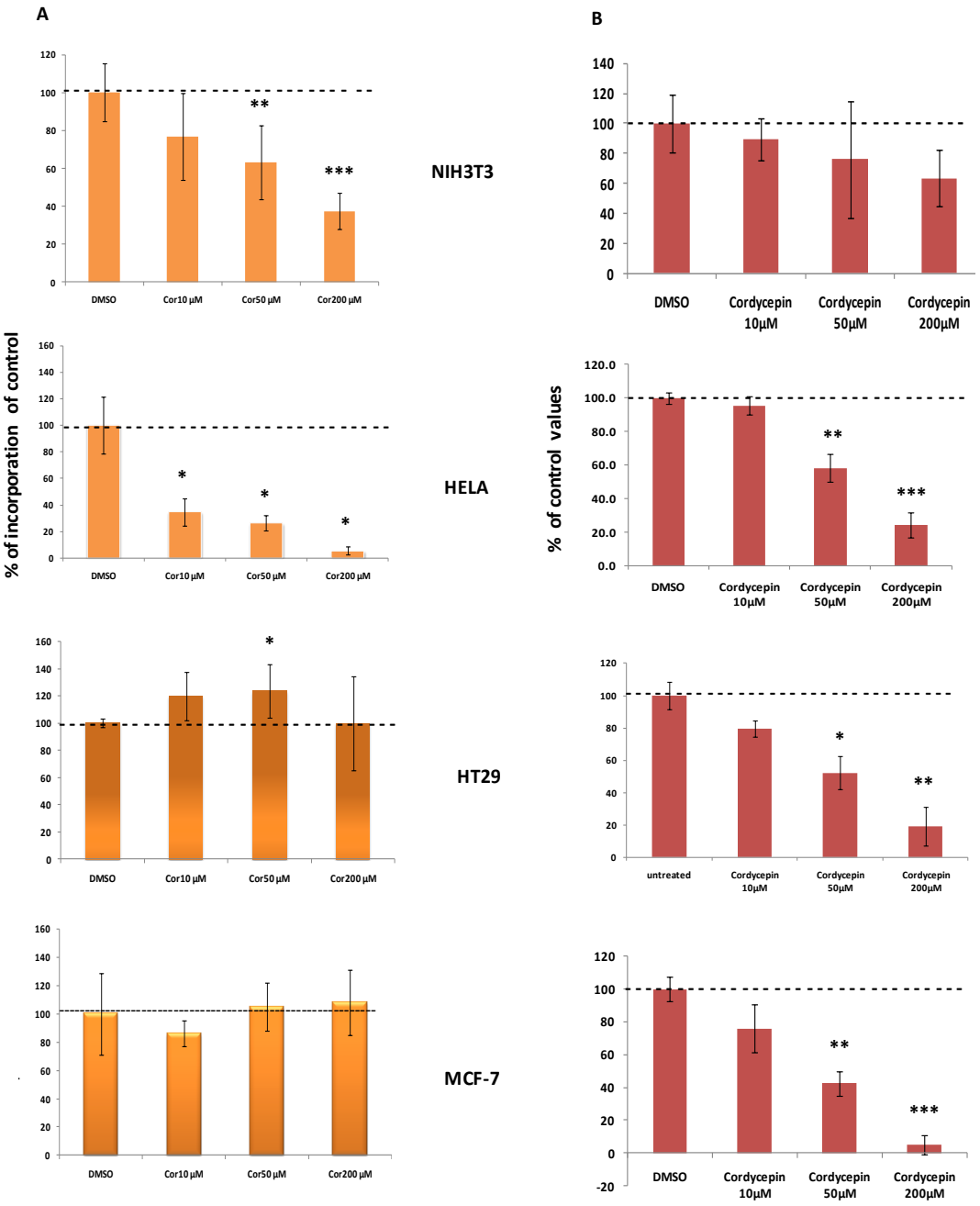
3 Characterizing the effects of cordycepin on different cell lines

In this chapter I investigate whether different cell lines react in the same manner to cordycepin or not by examining its effects on protein synthesis via mTOR pathway, 4-EBP phosphorylation and cell proliferation. To determine this, I have used cells treated with different doses of cordycepin and analysed them using various methods including ^{35}S incorporation, in-cell ELISA and counting of cells using trypan blue dye. This work further elucidates to the sensitivity of different cell lines to cordycepin and gives a better understanding of the broad spectrum of its action among various cell lines. I further investigated the stability issue of cordycepin in growth medium due to its degradation by adenosine deaminase over time in HELA and MCF-7 cells, using the adenosine deaminases inhibitor pentostatin. Pentostatin has significant toxicity, therefore I investigated effects observed using the minimal effective dose of pentostatin in cordycepin sensitive cell lines.

3.1 Dose response effect of cordycepin on protein synthesis by ^{35}S and ELISA on different cell lines

In order to analyse the effects of cordycepin on protein synthesis and 4-EBP phosphorylation by ^{35}S incorporation and in-cell ELISA, cells were treated with cordycepin at 10, 50 and 200µM for 2 hours. Our previous published work on NIH3T3 and HELA demonstrates dose response effects of cordycepin on the rate of protein synthesis (Wong et al. 2010) in figure 4 C and E. Previously, we had shown that sensitivity of the rate of protein synthesis to cordyepin increases with increasing dose of cordycepin. In Figure 3.1 A, the effect of cordycepin on cellular translation was measured by incorporation of radioactive amino acid for 10-15 min after a 2 hour cordycepin treatment. The rate of protein synthesis in NIH3T3 and HELA cells declines with an increasing dose of cordycepin, which was consistent from the previous published data for NIH3T3 and HELA cells (Wong et al. 2010). However, the rate of protein synthesis in HT29 and MCF-7 cells was insensitive to cordycepin, while HCT116 cells and A549 are affected by cordycepin to a lesser

extent. Statistical significance is shown as: * for P<0.05, ** for P<0.01 and *** for P<0.001.



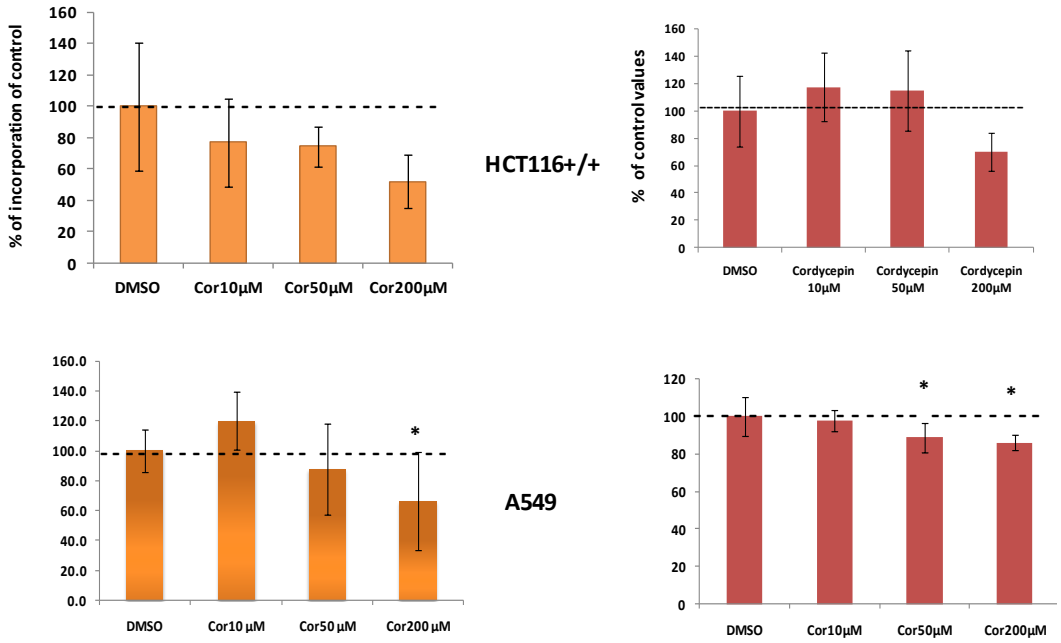


Figure 3.1: The sensitivity of cordycepin varies among different cell lines:

Indicated cell lines were treated with cordycepin for 2 hours at indicated concentrations. **A,** Protein synthesis rates were measured by incorporation of ^{35}S labeled amino acids into protein, corrected for total protein concentration and represented as a percent incorporation of the control. **B,** Levels of P- 4EBP were measured by in cell ELISA and expressed as percentages of the control. Experiments were performed in 4-5 technical replicates and the data are expressed as means ± S.D. For statistical significance unpaired T-test was performed in comparison with control (No cordycepin) indicated as * for P<0.05, ** for P<0.01 and *** for P<0.001.

In previously published data from our laboratory, it was shown that cordycepin inhibits protein synthesis in NIH3T3 cells by reducing 4EBP phosphorylation (Wong et al., 2010). To confirm the effects of cordycepin on the mTOR pathway levels of the mTORC1 product phospho- 4EBP were investigated by in cell ELISA. Levels of P-4EBP were examined in figure 3.1, B in different cell lines, which showed that P-4EBP levels decrease with an increasing dose of cordycepin in NIH3T3 cells in a 2 hour treatment, although a high variability makes this effect this only significant at the highest doses. Levels of P-4EBP in HELA cells are more sensitive to cordycepin. Surprisingly, HT29 and MCF-7 show marked decreases in the levels of P-4EBP with increasing doses of cordycepin, although their rate of protein synthesis remains insensitive. Levels of P-4EBP in HCT116 +/+ and A549 also remain unaffected. The effect of cordycepin on protein synthesis and phospho-4EBP levels varies therefore

among different cell lines, in some cases there is a strong correlation between the effect on phospho-4EBP levels and protein synthesis, but in others the effect is more subtle or absent. The validation of ELISA method for phospho-4EBP levels by western blotting is necessary, but this could not be achieved due to technical reasons. The data for the rate of protein synthesis and levels of P-4EBP are summarized in table 7.

Table 7: Summary of rate of protein synthesis and levels of P-4EBP by in cell ELISA on various cell lines:

Cell Line	Dose of cordycepin µM	Decline in rate of protein synthesis (%)	Reduction in P-4EBP levels (%)
NIH3T3	10	78	88
	50	62	78
	200	36	60
HCT116	10	78	110
	50	78	110
	200	48	78
MCF-7	10	82	74
	50	100	40
	200	100	4
HELA	10	32	100
	50	22	58
	200	2	22
HT29	10	110	80
	50	110	50
	200	98	18
A549	10	110	98
	50	98	88
	200	78	82

Summarized data expressed as percentages of the control values calculated from technical replicates.

3.2 The effects overtime of combining cordycepin with a stabilizing drug in MCF-7 cells

The effects of cordycepin on protein synthesis and phospho-4EBP levels may be limited by high levels of the cordycepin degrading enzyme adenosine deaminase in these cell lines. We examined the effects of Pentostatin (deoxycoformycin), an adenosine analogue which act as an adenosine deaminase inhibitor which

administered in combination with cordycepin may make it effective over longer time periods. MCF-7 cells were seeded a day before use, followed by a medium change and then treated with cordycepin (50µM) in absence or presence of pentostatin (1µM) for 2, 8 and 24 hours. As can be seen in figure 3.2, the relative rate of protein synthesis in comparison with untreated (no cordycepin, no pentostatin) was markedly decreased at 8 and 24 hours in combination with cordycepin with pentostatin as compared to the 2 hour time point. However the relative rate of protein synthesis with cordycepin in absence of pentostatin shows much less reduction at all indicated time points. There is also not much effect of pentostatin on its own compared to untreated cells. These data shows that pentostatin increases the effect of cordycepin over time in MCF-7 cells, presumably by inhibiting its degradation.

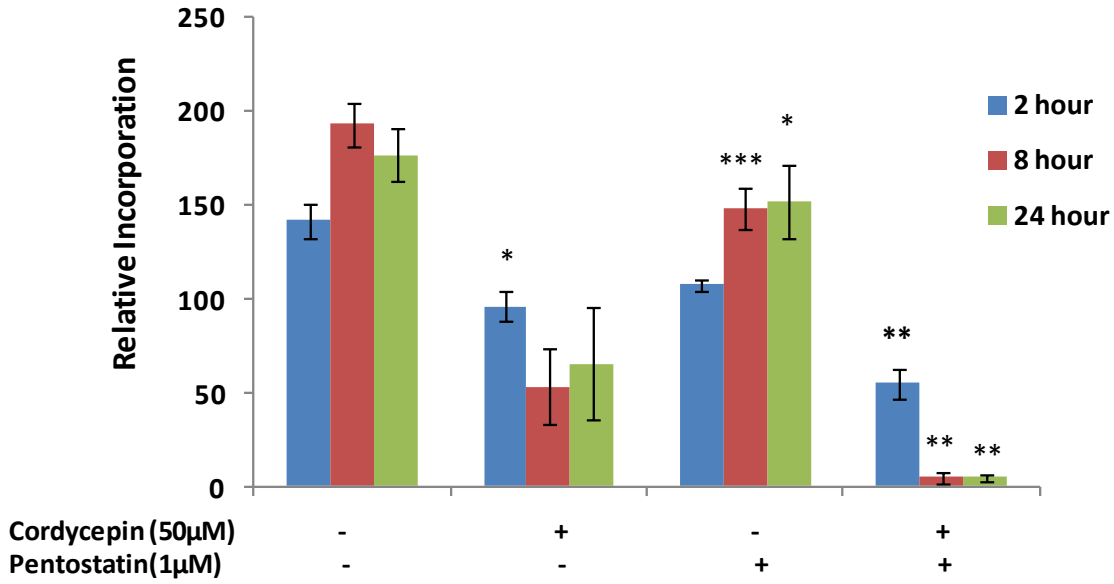


Figure 3.2: Effects of cordycepin and pentostatin on the rate of protein synthesis over time:

MCF-7 cells were treated with cordycepin (50µM) in absence or presence of pentostatin (1µM) for 2, 8 and 24 hours. Protein synthesis rates were measured by incorporation of ^{35}S labeled amino acids into protein, corrected for total protein concentration and represent as a relative incorporation. Error bar shows standard deviations over independent biological triplicates. For statistical significance Paired T-test was performed in comparison with control indicated as * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$.

The data showed in figure 3.3 represents cordycepin levels in the culture medium of MCF-7 cells (unpublished data, obatained in collaboration with Prof. Dave Barrete by Wahy Utami). MCF-7 cells were treated with cordycepin (50µM) in absence or presence of pentostatin (1µM) for 2, 8 and 24 hours. As can be seen in figure 3.3 that pentostatin stabilizes the concentration of cordycepin by slowly decreasing the level of cordycepin over time. The cordycepin disappears completely from the culture medium at 8 and 24 hours in the absence of pentostatin.

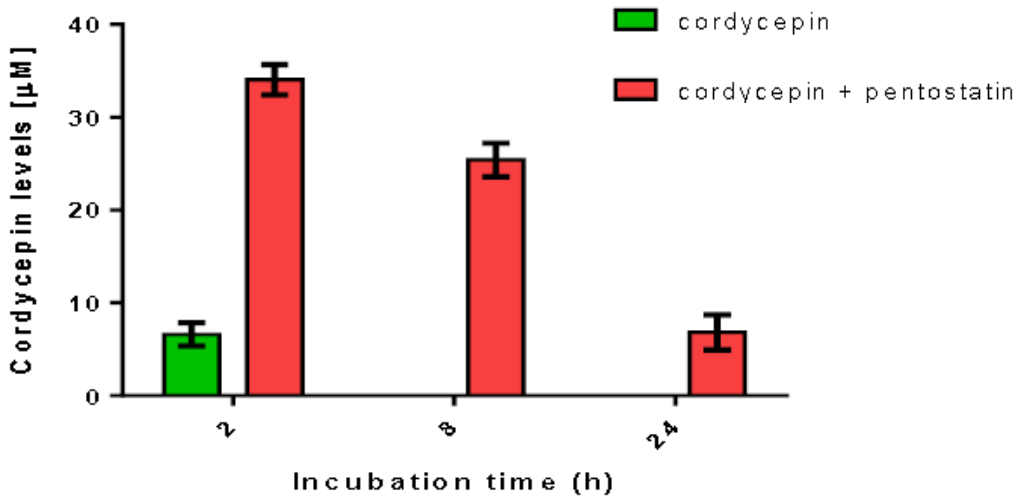


Figure 3.3: Pentostatin stabilizes cordycepin levels over time in MCF-7 cells:

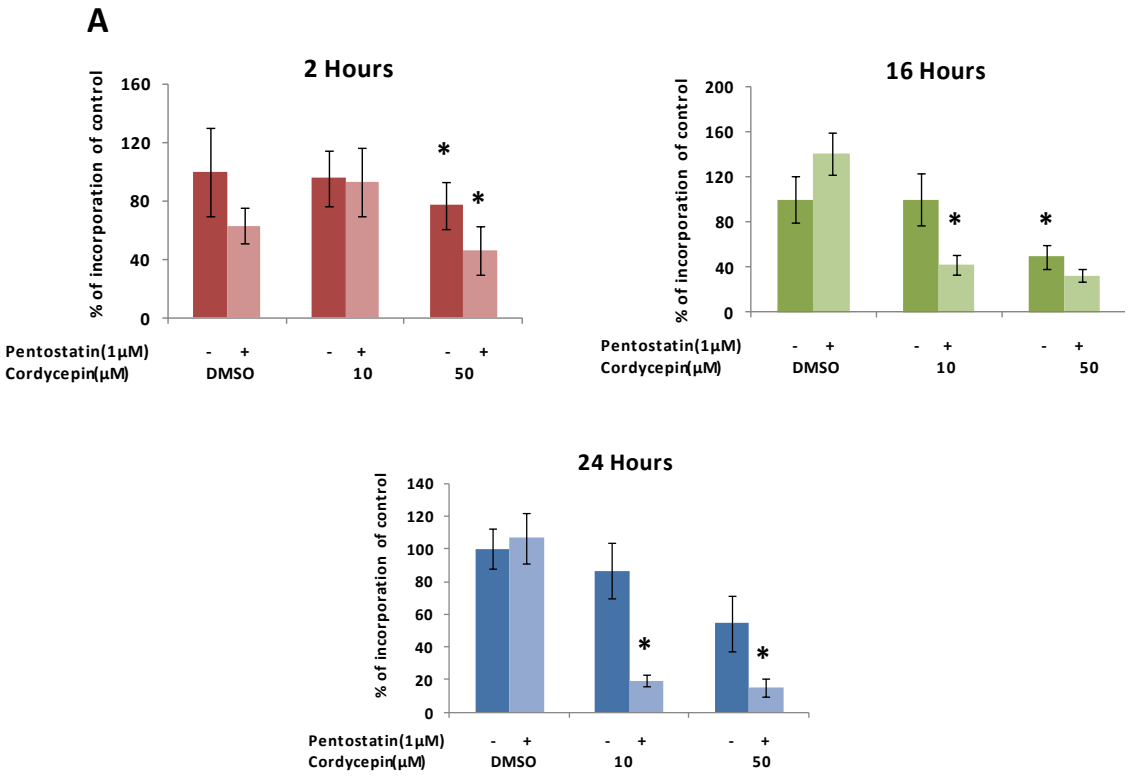
Concentrations of cordycepin were determined in the culture medium of MCF-7 cells. The cells were treated with cordycepin (50µM) in absence or presence of pentostatin (1µM) for 2, 8 and 24 hours. Each bar represents the mean ± SEM. (Work done by PhD student Wahyu Utami).

3.3 Pentostatin stabilizes cordycepin in HELA cells over time

Next, I investigated the long term effects of cordycepin on the cellular rate of translation and levels of P-4EBP in combination with pentostatin in HELA cells. HELA cells were treated with cordycepin with 10 and 50µM in presence or absence of pentostatin (1µM) for 2, 16 and 24 hours. It can be observed from figure 3.4A, that the rate of protein synthesis, represented in percentage incorporation of untreated (no cordycepin, no pentostatin), shows much more reduction at 2 hours with cordycepin (50µM) in presence of pentostatin compared with cordycepin alone. Cordycepin (50µM) decreases the rate of protein synthesis significantly at 16 and 24

hours in combination with pentostatin compared to on its own or at lower dose (10µM). These data demonstrate that pentostatin enhances the effects of cordycepin on the cellular rate of translation over time.

To assess whether the decline in rate of protein synthesis correlates with the levels of P-4EBP, we examined the HELA cells by in-cell ELISA for this effect, treated with cordycepin in combination with pentostatin (1µM) for 2, 16 and 24 hours. The levels of P-4EBP, represented as a percentage of the control values as seen in figure 3.4B, show clearly that pentostatin enhances the effect of cordycepin on 4EBP phosphorylation at all timepoints, although P-4EBP levels appear to recover somewhat in the later timepoints. Validation of the ELISA method for phospho-4EBP levels by western blotting is necessary but this could not achieve due to technical reasons.



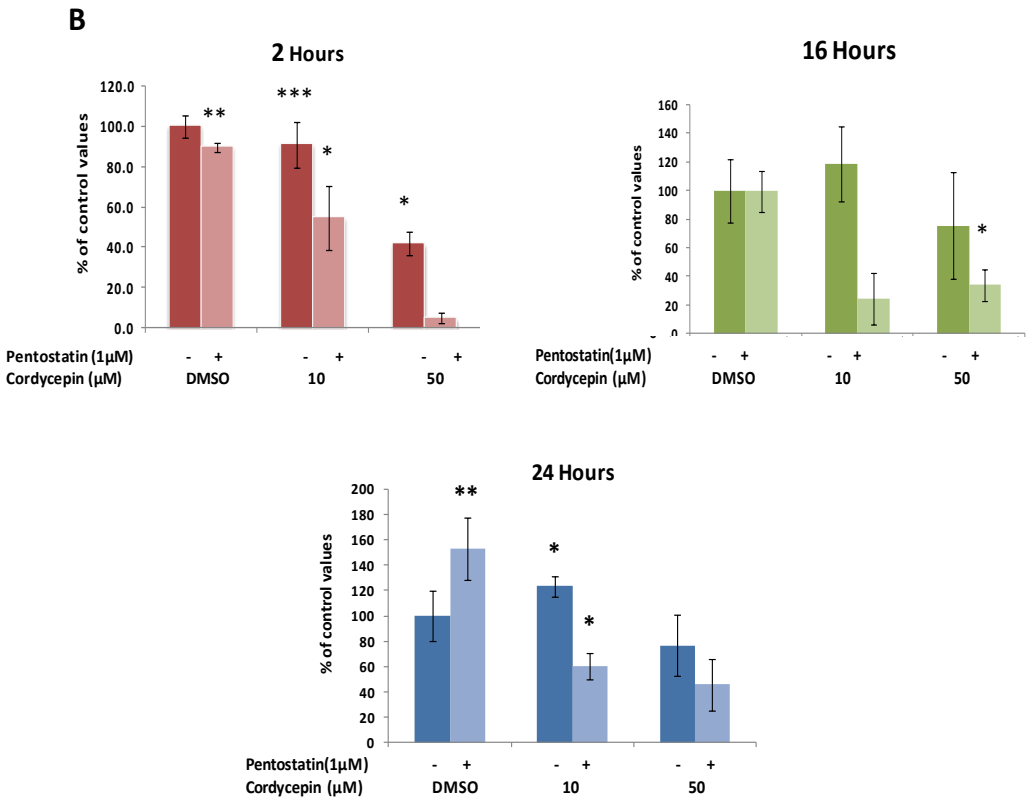


Figure 3.4: Pentostatin increases the effect of Cordycepin in HELA:

HELA cells were treated with cordycepin in absence or presence of pentostatin at indicated concentrations for 2, 16 and 24 hours. **A,** Protein synthesis rates were measured by incorporation of ^{35}S labeled amino acids into protein, corrected for total protein concentration and represented as a of percent incorporation of control. All error bars represents standard deviations over three independent biological replicates. A paired T-test was performed and stars indicate significance: * for P<0.05, ** for P<0.01 and *** for P<0.001. **B**, Level of P-4EBP was measured by in cell ELISA for 2, 16 and 24 hours. Reduction in levels of P-4EBP without pentostatin seems to be much less compared with in presence of pentostatin. Values are expressed as percentages of the control. Error bars are representative of results obtained from four technical replicates. Unpaired T-test was performed to show statistical differences among treatment represents as * for P<0.05, ** for P<0.01 and *** for P<0.001.

3.4 A minimal dose of pentostatin is still effective

As we have seen that co-administration of pentostatin with cordycepin stabilizes and improves its efficacy, there is a need to investigate any undesirable side effects of pentostatin that have been recorded both in patients and in animal models (Airhart et al. 1996, Lauria and Forconi 2009, Tadmor 2011). In order to reduce its toxicity we investigated the minimal effective concentration of pentostatin in NIH3T3 cells by its effects on cellular rate of protein synthesis. NIH3T3 cells were

titrated with different concentrations of pentostatin from 1000nM - 1.25nM in presence or absence of cordycepin (50µM) for 16 hours.

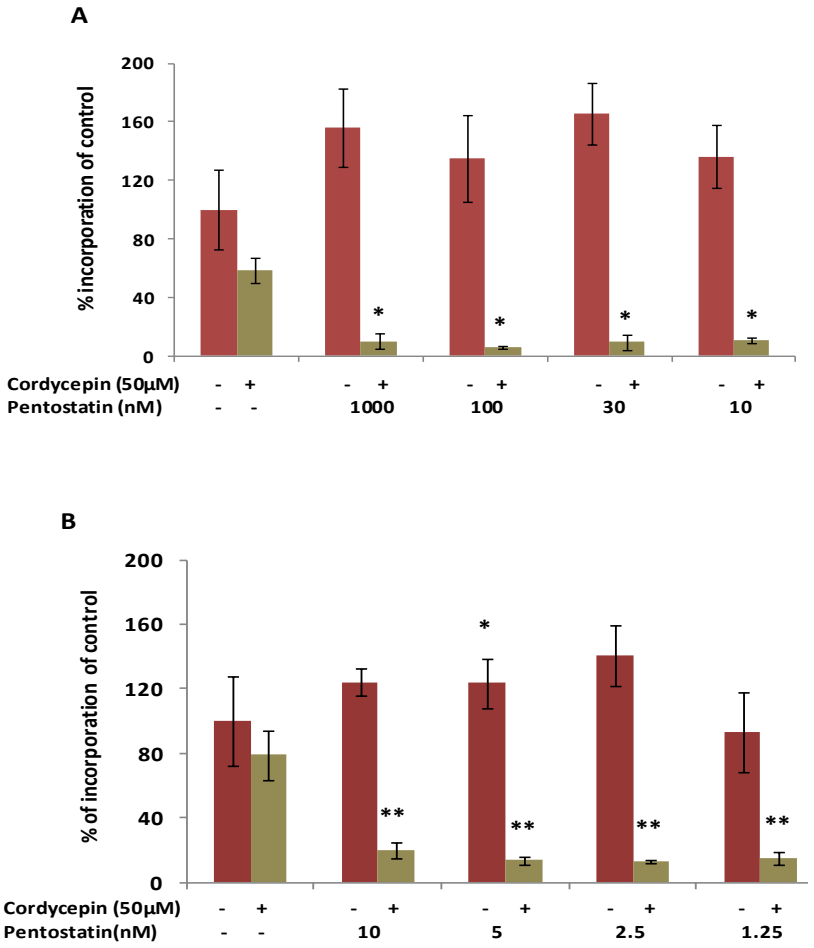


Figure 3.5: Concentration titration of pentostatin in combination with cordycepin:

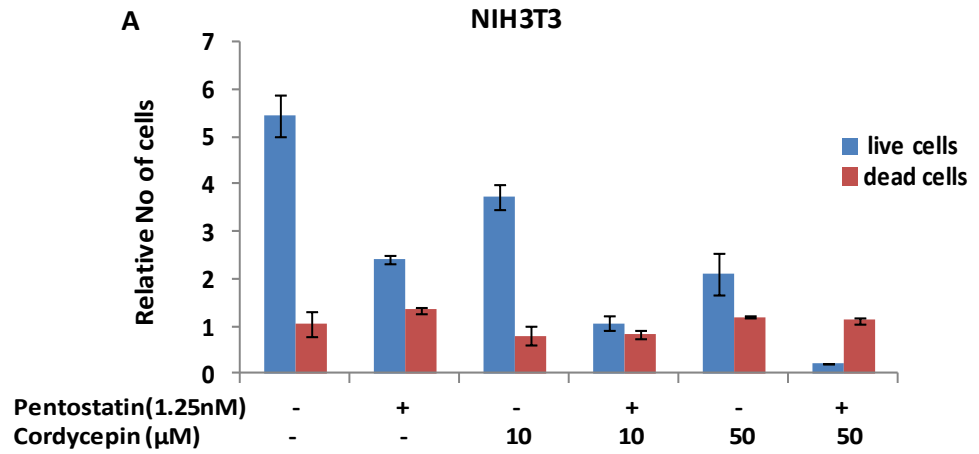
A & B, NIH3T3 cells were treated with different concentrations of pentostatin as indicated in presence or absence of cordycepin (50µM) for 16 hours. Protein synthesis rates were measured by incorporation of ^{35}S labeled amino acids into protein, corrected for total protein concentration and represent as a percent incorporation of control. All error bars represents standard deviations over four to five technical replicates. Unpaired T-test for a statistical significance in comparison with control (No cordycepin) indicated as * for $P<0.05$ and ** for $P<0.01$.

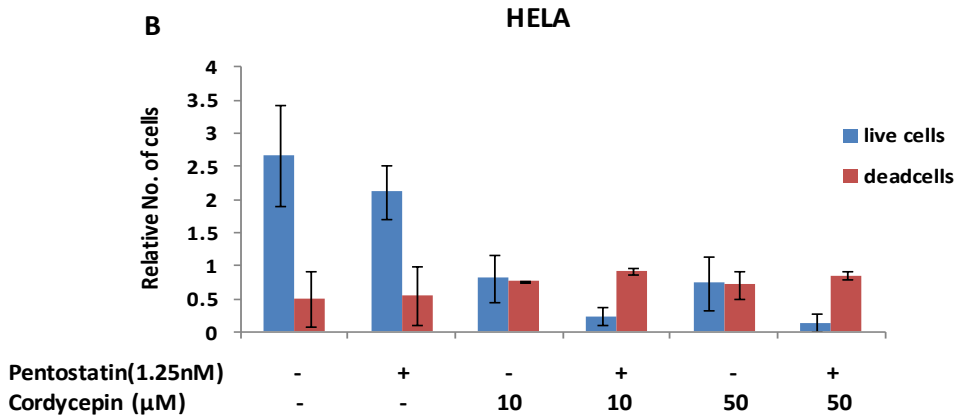
Rate of protein synthesis with different doses of pentostatin (1000nM - 1.25nM) in combination with cordycepin seems to be sensitive as shown in figure 3.5A and 3.5B, compared with pentostatin alone which seems to be insensitive with effects on cellular rate of translation. For a significant statistical difference, effects of protein synthesis need to be examined on more biological replicates. We can conclude that doses of pentostatin of 1.25 nM are still very active in combination

with cordycepin and are likely to reduce its undesirable effects in vivo to a greater extent.

3.5 Cell proliferation and survival are affected by cordycepin in different cell lines

In a few cell lines we have seen effects on protein synthesis with changes in levels of P-4EBP while in most cell lines we observed that cordycepin is affecting either one of these. Now we need to investigate whether the sensitivity of cordycepin for cellular rate of protein synthesis combined with levels of P-4EBP is related to its effects on cell proliferation and survival. NIH3T3, HELA, MCF-7 and A549 cells were treated a day after seeding with cordycepin at 10μM and 50μM while HCT116+/+ was treated only at 50μM in absence or presence of pentostatin (1.25nM) for 72 hours and undergo cell counting by using trypan blue dye as (dead) blue and (live) white cells. As shown in figure 3.6, cell numbers were decreased by treatment with cordycepin (50μM), and this was enhanced in the presence of pentostatin (1.25nM) in NIH3T3, HELA, MCF-7, HCT116+/+ and A549 cells. Cell proliferation is also reduced with a lower concentration of cordycepin (10μM) but to a lesser extent than with higher concentrations (50μM) in these cell lines, although it is not always statistically significant. In HCT116 and MCF-7 cells cordycepin and pentostatin appear to primarily inhibit cell division. Pentostatin alone also inhibits cell proliferation in NIH3T3. However pentostatin does not inhibit cell proliferation in HCT116 and MCF-7. This indicates that different cell lines have different sensitivities to pentostatin. The sensitivity of NIH3T3 cells for cell proliferation by cordycepin is consistent with the previous published work (Wong et al., 2010) shown in figure 1D for NIH3T3 cells. These results indicate that co-administration of pentostatin with cordycepin increases the anti proliferative effects over longer period of times.


A
NIH3T3
Relative No of cells
0
1
2
3
4
5
6
7
live cells
dead cells
Pentostatin(1.25nM) - + - + - +
Cordycepin (µM) - - 10 10 50 50


B
HELA
Relative No. of cells
0
0.5
1
1.5
2
2.5
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3.5
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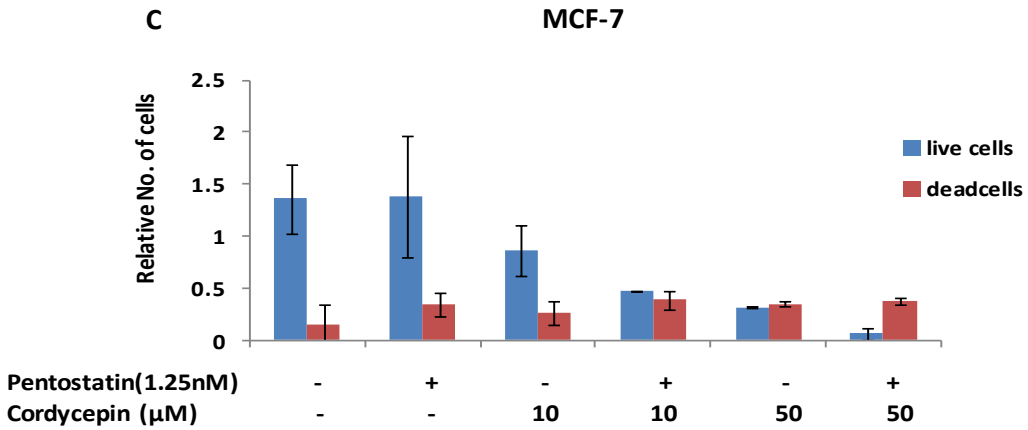
live cells
deadcells
Pentostatin(1.25nM) - + - + - +
Cordycepin (µM) - - 10 10 50 50


C
MCF-7
Relative No. of cells
0
0.5
1
1.5
2
2.5
live cells
deadcells
Pentostatin(1.25nM) - + - + - +
Cordycepin (µM) - - 10 10 50 50

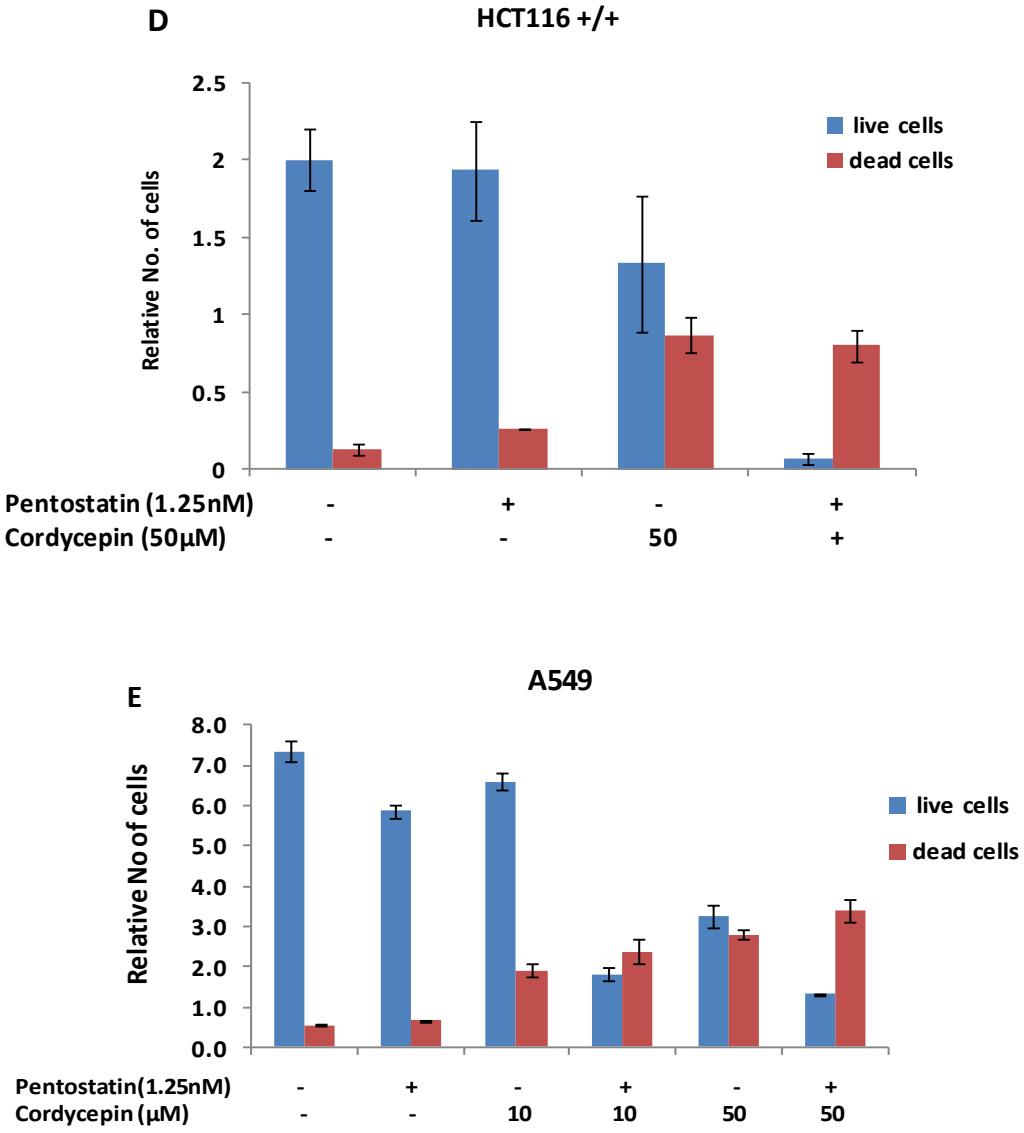


Figure 3.6: Pentostatin enhances the long-term effects of cordycepin:

Indicated cells were treated with cordycepin with indicated concentrations in absence or presence of pentostatin (1.25nM) for 72 hours and cell counts were done by counting (dead) blue and (live) white cells using trypan blue dye under microscope. Pentostatin co-administration stabilized and improved the anti-proliferative effect of cordycepin. Results are presented as a relative number of cells and data represents standard deviations over two independent biological replicates for each treatment.

3.6 Effects of low dose of pentostatin on cell proliferation

After observing effects on the cellular rate of translation at low doses of pentostatin in presence of cordycepin (50µM), we further titrated down the dose of pentostatin to determine the least minimal effective dose of pentostatin on cell proliferation in NIH3T3 cells in combination with cordycepin (5µM). NIH3T3 cells were treated with different doses of pentostatin (1nM - 0.025nM) in combination with cordycepin (5µM) for 72 hours followed by counting the cells in trypan blue dye under the microscope. As can be seen from figure 3.7, cell numbers declined to a much greater extent at all low doses of pentostatin in the presence of cordycepin compared to cordycepin (5µM) alone, which only has a small effect on cell proliferation. Therefore pentostatin seems to be significantly effective at very low doses and combination of pentostatin with cordycepin not only stabilizes its activity but also increases its anti-proliferative properties.

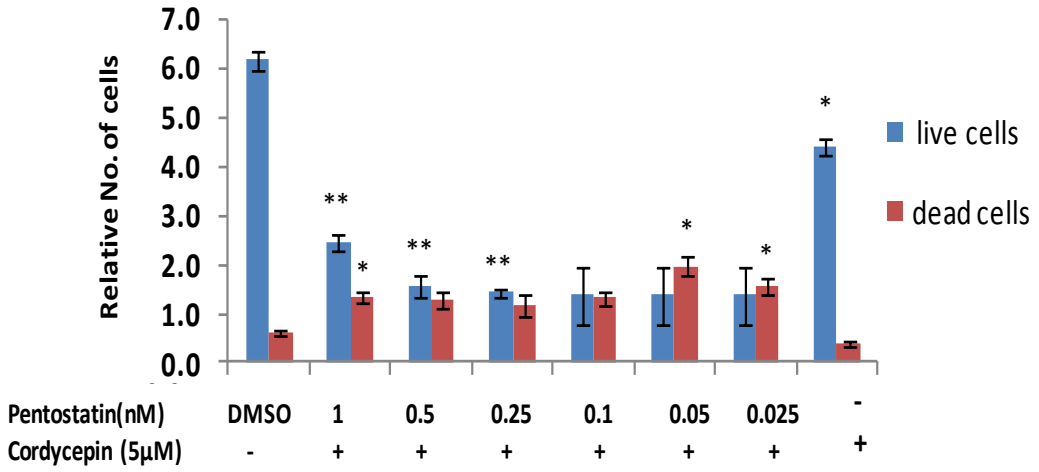


Figure 3.7: Dose response effects of pentostatin with cordycepin on cell proliferation:

NIH3T3 cells were treated with indicated concentration of pentostatin in presence of cordycepin (5µM) for 72 hours and cell counts were done by counting (dead) blue and (live) white cells using trypan blue dye under microscope. Results are presented as a relative number of cells and experiments were performed in four to five technical replicate expressed as means ±S.D. Unpaired T-test was performed to show statistical significance compared to untreated control represents as * for P<0.05 and ** for P<0.01.

3.7 Minimal doses of pentostatin are still effective at lower doses of cordycepin

We next want to examine the minimal effective dose of cordycepin in combination with lowest active dose of pentostatin in MCF-7 cells in order to reduce the possibility of any undesirable effects due to any of these drugs. Cells were treated with different low doses of cordycepin (2µM- 8µM) in combination with pentostatin at 1.25nM for 72 hours. The cells were counted using trypan blue dye and cell numbers are shown relative to the seeding density of the cells. It can be observed from figure 3.8 that cell numbers seem to be reduced by half at the lowest tested dose of cordycepin in presence of pentostatin compared to higher dose of cordycepin at 4µM and 8µM, which shows a bigger decline in cell numbers in a dose dependent manner. Effects on cell proliferation on live and dead cells were significantly different indicated as * for $P<0.05$ and ** for $P<0.01$ performed by unpaired t- test.

Therefore, it can be concluded that cordycepin is still very active and stable at its lowest dose, as it is reducing cell numbers in combination with low dose of pentostatin. The addition of pentostatin also improves its efficacy by reducing its toxicity over time in medium. We conclude that its instability reduces the biological activity of cordycepin significantly and that this is likely to affect its usefulness for cancer therapy. The data indicate that when administrated in combination, much lower doses of cordycepin and pentostatin can be used than previously reported.

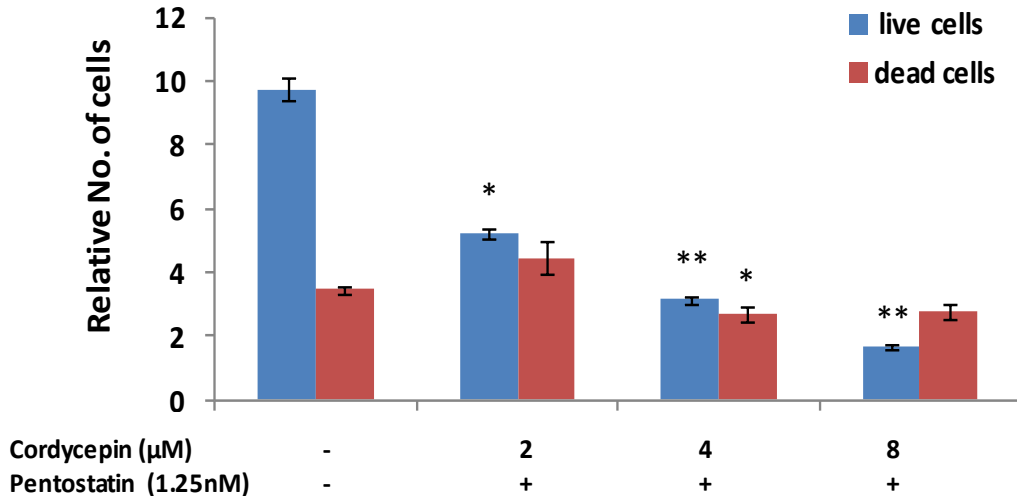


Figure 3.8: Effects of low doses of cordycepin and pentostatin on cell proliferation in MCF-7*:*

MCF-7 cells were treated with indicated concentrations of cordycepin in presence of pentostatin (1.25nM) for 72 hours and cell counts were done by counting (dead) blue and (live) white cells using trypan blue dye. Results are presented as relative numbers of cells. Measurements were performed in 4-5 technical replicate expressed as means ±S.D. Statistical significance in comparison with control (No cordycepin) were determined by unpaired T-test indicated as * for P<0.05 and ** for P<0.01.

3.8 Discussion

So far we have seen that effects of cordycepin are cell type specific and that the sensitivity of cordycepin towards protein synthesis and changes in levels of P-4EBP varies among different cell lines. Except MCF-7 and HT29 cells, all examined cell lines are sensitive to cordycepin in their rate of protein synthesis while HCT116 is affected to a much lesser extent. However, A549 does not show much change in the rate of protein synthesis or changes in levels of P-4EBP ELISA. The dose response effects on protein synthesis in NIH3T3 and HELA cells are similar to those observed previously (Wong et al., 2010). However levels of P-4EBP are sensitive to cordycepin in HELA, HT29 and MCF-7 cells and to a lesser extent in NIH3T3 cells. Cordycepin does not significantly change the levels of P-4EBP in HCT116+/+ and A549 cells. In NIH3T3 and HELA cells, the rate of protein synthesis and levels of P-4EBP are sensitive to cordycepin. The data on 4EBP phosphorylation for MCF-7 cells are puzzling as they indicate that protein synthesis was unaffected in these cells,

however there is still a prominent reduction in 4EBP phosphorylation. This could be due to a reduction in total 4EBP levels in MCF7 cells in response to cordycepin, however it was not possible to measure changes in total 4EBP levels by western blotting due to technical reasons. Such an effect has been observed by Western blot in NIH3T3 cells (Wong et al, 2010). To check this it would be necessary to measure total 4EBP levels as well as phospho-4EBP levels. Another potential cause of the differences between cell types could lie in the decay rates of the drug in absence of pentostatin. We have seen complete disappearance of cordycepin levels in the medium at 8 and 24 hours on its own with only a small amount of cordycepin left at 2 hours. However, a gradual drop in the level of cordycepin has been observed when cordycepin (50µM) is used in combination with pentostatin (1µM) (unpublished data, obtained in collaboration with Prof. Barrett by Wahyu Utami). A similar pattern was observed for the levels of cordycepin triphosphate inside the cell, which persist longer in the presence of pentostatin. Previously (Wong et al., 2010), it was demonstrated that cordycepin reduced AKT kinase and 4E- BP phosphorylation by the mTORC1 and mTORC2 complexes and activates AMP activated kinase (AMPK) by an unidentified mechanism. Factors which the make sensitivity of cordycepin variable among different cell lines need to be investigated in future.

Protein synthesis is strongly affected by cordycepin with pentostatin at later time points in HELA and MCF7 cells. This is likely to be through its effects on cell survival, as subsequent work has demonstrated that the combination of cordycepin with pentostatin induces apoptosis within 8 hours (Richa Singhania, unpublished result). I have shown that pentostatin potentiates the effects of cordycepin, even with very low doses of both. Given the known immune suppressive effects of pentostatin *in vivo,* it is likely that optimal therapy will need to employ its lowest active dose, which appears to be nearly a thousand fold lower than the doses commonly used in the clinic.

We have seen that cordycepin inhibits proliferation in all cell lines, regardless of its effects on 4EBP phosphorylation and protein synthesis. Therefore, we can conclude

that effects on protein synthesis via inhibition of mTOR are not always responsible for the effects of cordycepin on cell proliferation.

4 Effects of cordycepin on gene expression, polyadenylation and cell adhesion in MCF7 cells

In order to be able to design and validate more stable analogues or formulations of cordycepin, we first have to understand the mechanism of action of the drug. As outlined in the introduction, cordycepin is certainly a polyadenylation inhibitor, but other mechanisms of action have also been proposed to contribute to the therapeutic properties of cordycepin. In this chapter we study the effects of cordycepin observed previously in other cell lines, in more detail in MCF-7 cells. We examined the MCF-7 cells in detail because they were found to be insensitive at the level of protein synthesis but exhibit significant sensitivity on cell proliferation. We can therefore discount any indirect effects due to reduced protein synthesis at the earlier timepoints, where the primary effects must be taking place. We examined the effects of cordycepin on gene expression, cell adhesion and polyadenylation.

4.1 Inflammatory mRNAs affected by cordycepin

In order to narrow down the events leading to the reduction in proliferation that is induced by cordycepin, I decided to try to identify the earliest effects on gene expression. In the first instance, I pursued a candidate gene approach. The TWIST1 mRNA was selected as it is regulated by CPEB and found to be upregulated in epithelial-mesenchymal transition in MCF-10A and it shows sensitivity towards cordycepin (Nairismagi et al. 2012). I was further interested in investigating the sensitivity of inflammatory mRNAs to cordycepin (PTGS2, CCL2, CXCL1 and IL8) in MCF-7 cells, because we have previously shown they are sensitive in primary airway smooth muscle cells (Kondrashov et al; 2012). Cells were treated 24 hours after seeding for 2 hours with cordycepin (50μM). The cells were harvested and total RNA was isolated as described previously. RNA was reverse transcribed and subjected to RT-qPCR. As a control, the housekeeping mRNAs encoding ribosomal protein (RPL10A) and β actin (ACTB) were used. Normalization was performed to GAPDH, a

well characterised house-keeping gene. It can be observed from figure 4.1 that cordycepin reduces the expression of IL8 and CXCL1. Other inflammatory mRNAs e.g PTGS2 and CCL2 were not significantly affected. The expression of TWIST1 mRNA was also greatly reduced by cordycepin in MCF-7 cells.

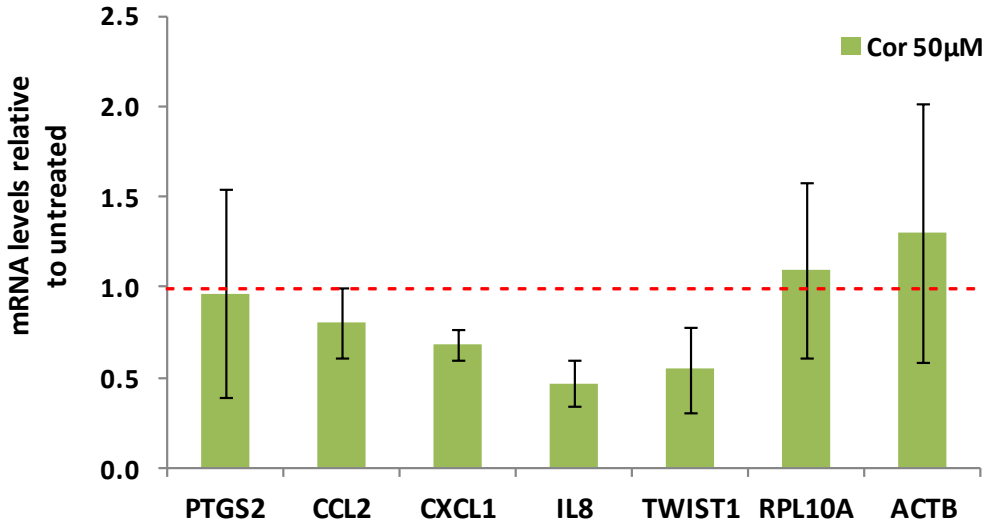


Figure 4.1: Levels of inflammatory mRNAs affected by cordycepin:

Total RNA was isolated from MCF-7 cells treated with cordycepin (50µM) for 2 hours. RT-qPCR was performed for mature mRNAs and the mRNA levels were expressed as normalised to GAPDH mRNA. Untreated control values (no cordycepin) were set at 1. All error bars are standard deviations over three independent biological replicates. Statistical significance compared to control (No cordycepin) were determined by Paired T-test indicated but none of the examined mRNA found to be statistically different from the untreated.

4.2 Cordycepin acts as a polyadenylation inhibitor

After examining the effects of cordycepin on protein synthesis, levels of P-4EBP and on cell proliferation in MCF-7 cells, we investigated whether cordycepin acts as a polyadenylation inhibitor in MCF-7 cells. To examine this MCF-7 cells were treated with increasing concentrations of cordycepin 10µM and 50µM to study the effects on total poly(A) tail length. MCF-7 cells were treated for 2 hours with cordycepin followed by total RNA isolation. RNA was subjected to 3' end labeling and non-poly adenylated RNA was degraded with RNAses A and T1 followed by electrophoresis on urea polyacrylamide gel. It can be observed from figure 4.2 that 10 µM and 50 µM cordycepin equally reduce the total poly(A) tail length in the cell.

Figure 4.2: Dose response effect of cordycepin on total poly (A) tail length in MCF-7:

MCF-7 cells were treated for 2 hours with cordycepin at indicated concentrations. Total RNA was prepared and labelled at 3´ end with ^{32}P-pCp along with other necessary components and non-polyadenylated RNA degraded, resulting in a smear representing the total poly (A) mRNAs in the cell. The reaction products were analyzed by urea (7M) 15% Polyacrylamide gel electrophoresis. Labelled RNA ladder was used as a marker where each size corresponds to different poly(A) tail lengths in bases.

4.3 Polyadenylation is affected by cordycepin over time

As we demonstrated that cordycepin at both 10 and 50 μM reduces the length of total poly(A) tails in MCF-7 cells after 2 hours, we next examined whether pentostatin mediated stabilization of cordycepin, resulting in the inhibition of polyadenylation for a longer time period. MCF-7 cells were treated with cordycepin in the absence or presence of pentostatin for 2, 8 and 24 hours followed by total RNA isolation. RNA was subjected to 3' end labeling followed by degradation of non-polyadenylated RNA with RNAses A and T1 and electrophoresis on urea polyacrylamide gel. It can be observed from figure 4.3 that total poly(A) tail length is reduced equally by cordycepin (50μM) and cordycepin with pentostatin (1 μM) at 2 hours and the same effects were also observed at the 8 hour time point. However at 24 hours the total poly(A) tail size is recovered in the presence of cordycepin alone.

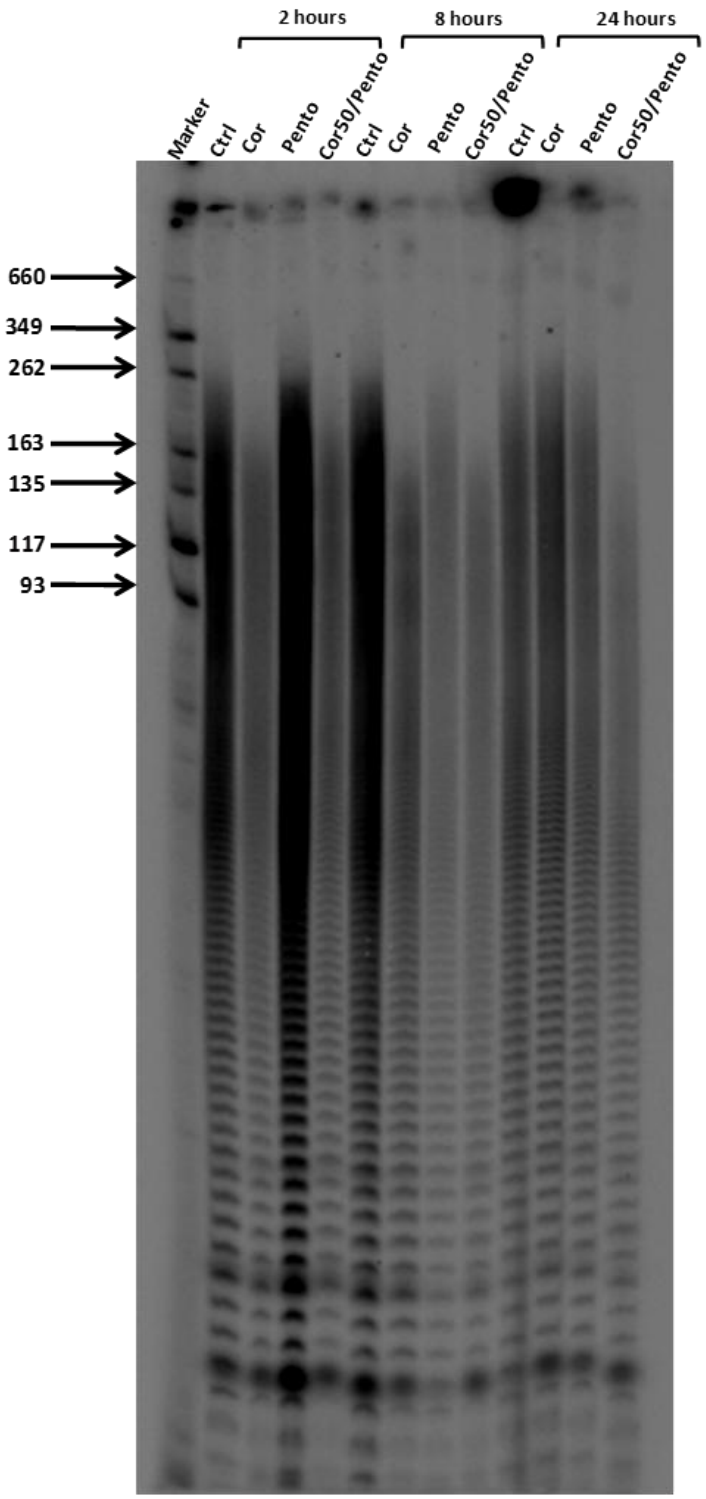


Figure 4.3: Effects of cordycepin on total poly (A) tail length over time in MCF-7:

MCF-7 cells were treated with cordycepin (50µM) in absence or presence of pentostatin (1µM) for indicated time points. Total RNA was prepared and labelled at the 3´ end with ^{32}P-pCp along with other necessary components and non-polyadenylated RNA degraded which results in a smear presenting the total poly(A) mRNAs in the cell. The reaction products were analyzed by urea (7M) 15% Polyacrylamide gel electrophoresis. A labelled RNA ladder was used as a marker where each size corresponds to different poly(A) tail lengths in bases.

4.4 Effects on cell adhesion by cordycepin in MCF-7 cells

It was demonstrated previously in NIH3T3 cells that cells lost focal adhesions upon treatment with cordycepin at 50µM (Wong et al., 2010). As we had already seen effects of cordycepin on polyadenylation and cell proliferation we next examined the effects cordycepin had on cell shape. In order to study changes in cell shape in detail, immunohistochemistry was performed using the focal adhesion marker vinculin. MCF-7 cells were seeded on cover slips and were treated after 24 hours with cordycepin 50µM for 3 hours. As observed in figure 4.4, no changes were observed in focal adhesions in MCF-7 cells after treatment with cordycepin 50µM.

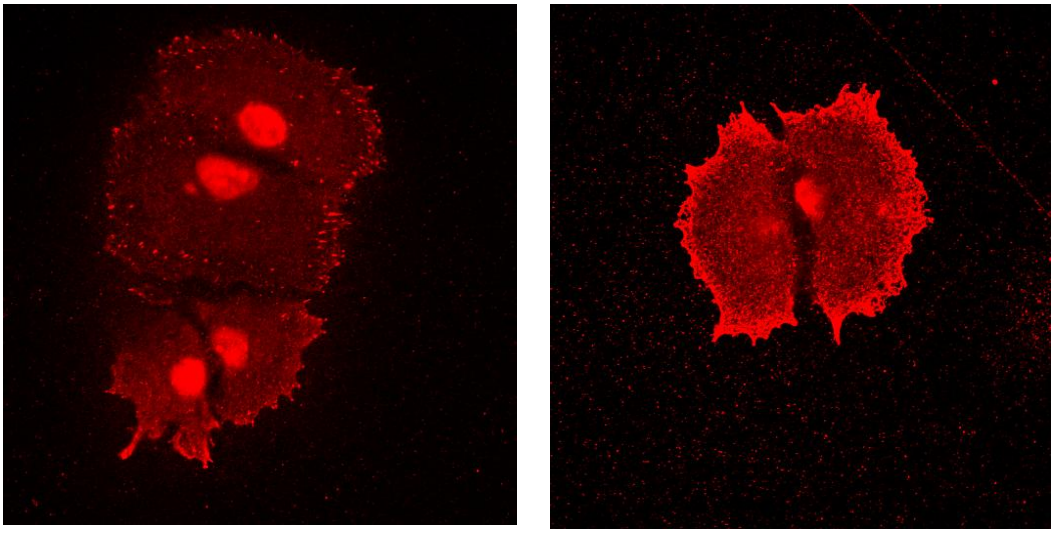


Figure 4.4: Immunohistochemistry of MCF-7 cells treated with cordycepin:

On coverslips MCF-7 cells were seeded the day before treatment. Next day cells were treated for 3 hours with cordycepin (50µM). The cells were fixed and stained with an antibody against vinculin (red) to detect focal adhesions. Images were taken at 63X magnification.

4.5 Discussion

In this chapter we have shown that like in ASM cells, gene expression of inflammatory mRNAs (CXCL1 and IL8) in MCF-7 cells are sensitive to cordycepin. However control mRNAs which are more abundant and stable (RPL10A and ACTB) remain unchanged. This indicates that effects on inflammatory mRNAs by cordycepin are not cell type specific but seem to be gene type specific. Cordycepin

10µM already inhibits polyadenylation compared to 50µM which clearly indicates that cordycepin represses polyadenylation in MCF7 cells. Therefore it can be concluded that as in ASM cells, cordycepin also inhibits the gene expression of several inflammatory mRNAs in MCF-7 cells. TWIST1 was also found to be downregulated in MCF-7 cells, same as previously observed in MCF-10A cells.

Pentostatin, an adenosine deaminases inhibitor, enhances the potency of cordycepin, presumably by preventing its degradation and improves its efficacy over time. We demonstrate that at the 2 and 8 hour time points Polyadenylation is equally affected by cordycepin in the absence or presence of pentostatin however poly(A) tail size recovers after 24 hours in the absence of pentostatin. The lack of recovery in the cells treated with cordycepin and pentostatin is likely to be due to the death of the cells by apoptosis (Richa Singhania, personal communication).

We have previously seen effects on cell adhesion in NIH3T3 cells, but in MCF-7 cells no changes in focal adhesions were detected by immunohistochemistry. We did observe changes in cell shape under the microscope in tissue culture dishes, but these changes seem to have vanished when we plated the cells on glass coverslips to perform immunohistochemistry in detail. This might be due to the difference in the adhesion surface, the seeding density or the time in culture after seeding. These parameters may have to be re-examined in the future.

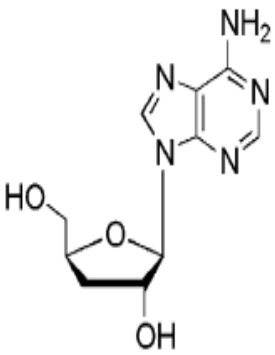
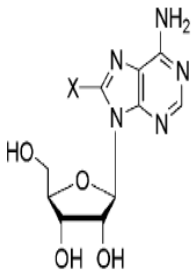
5 Investigating the mechanism of action of cordycepin in MCF-7

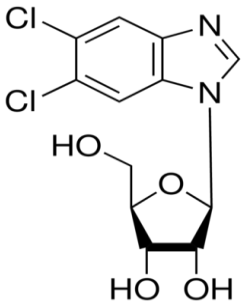
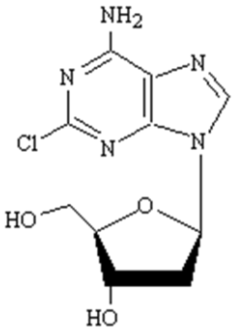
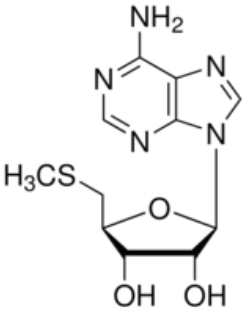
In this chapter I will examine the mode of action of cordycepin in detail in MCF-7 cells. As observed previously, the stability of cordycepin improves when used in combination with pentostatin over time, however adverse effects of pentostatin have been reported. Other adenosine analogues which inhibit polyadenylation might therefore be better drugs than cordycepin. Adenosine analogues with their mode of action and further details were listed in table 8. However, we will have to determine if such compounds have similar mechanisms of action to cordycepin by

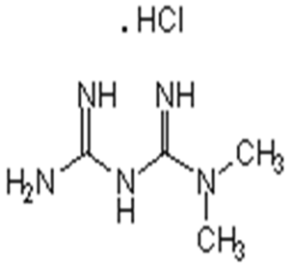
also examining their effects on protein synthesis, 4EBP phosphorylation and effects on inflammatory mRNAs and polyadenylation, which could be more stable than cordycepin.

Table 8: Description of adenosine analogues and related compounds:

Chemical structure, mode of action and biological effects were summarized for all examined adenosine (ado) analogues and related compounds (Metformin).

Adenosine analogues	Chemical Structures	Biological effects	Mode of action
Cordycepin		Medicinal properties of cordycepin range from anti-bacterial and anti-fungal to anti-malarial, antivirus, anti-harpes, anti-leukemic and anti-diabetic (Shin et al. 2009). Pharmacological activities such as anti-metastatic, anti-oxidant and immunomodulatory effects by suppression of over expressed inflammation (Jeong et al. 2010). Antiapoptotic reduces cell proliferation and metastasis, inhibits cell signalling, pathways (He et al. 2010) and have anti-tumor activity in colon, leukemia, bladder, and lung carcinoma cells (Yoshikawa et al. 2008). Lipid peroxide formation inhibition (Buenz et al. 2005).	Premature termination of chain by inhibiting polyadenylation (Kondrashov et al; 2012)
8 Bromo ado **8 Chloro ado** **8 Amino ado** **8 Azido ado**	 **C8 Ado** **X=Br,Cl,NH_2,N_3**	In multiple myeloma cells, mantle cell lymphoma, breast cancer cell lines and in primary CLL lymphocytes decline in ATP pool observed (Dennison et al. 2009)	Polyadenylation inhibitor, chain termination leads to inhibition of RNA synthesis (Chen et al. 2010)

DRB or 5,6-Dichloro-1-β-D-ribofuranosyl benzimidazole		Induce DNA damage pathway leads to arrest at G1 phase in cancer cells (Turinetto et al. 2009).	Affects polyadenylation by reducing the recruitment of polyadenylation factors to RNA polymerase II(Glover-Cutter et al. 2008) , act as inhibitors of transcriptional CDK7 and CDK9 the kinases involves in phosphorylation of C-terminal domain (CTD) of RNA polymerase II (Chen et al. 2009, Turinetto et al. 2009, Mapendano et al. 2010).
Se13 and Se15	Unknown (gifted)	May act same as DRB	cdk9 inhibitor could interfere in polyadenylation
Cladribine or 2-Chloro-2'-deoxyadenosine		Used in hairy cell leukemia, cell cycle arrest and induces apoptosis	Inhibits DNA repair, DNA synthesis and aggregation of DNA strand breaks (Robak and Robak 2012). Interferes in RNA synthesis, also inhibits a poly(A) polymerase activity (Chen et al. 2008)
5'-Deoxy-5'-(methylthio) ado		Act as an anticancer agent (Christopher et al. 2002, Avila et al. 2004), inhibitor of cell proliferation (Christa et al. 1984)	By-product of polyamine metabolism, 5'-methylthioadenosine phosphorylase (MTAP) a tumor suppressor gene involved in purine salvage pathway. (Christopher et al. 2002, Bertino et al. 2011)

Metformin or 1,1-Dimethylbiguanide hydrochloride		Anticancer drug in animals used in bowel cancer, anti diabetic, lower the chance of cancer in patients suffering with type 2 diabetics (Libby et al. 2009, Martin-Castillo et al. 2010, Loubiere et al. 2013).	AMPK activator (Dowling et al. 2007), similar cellular effects to cordycepin.

If cordycepin affects cells primarily by its properties as a polyadenylation inhibitor, unrelated compounds that affect polyadenylation should have similar effects. We therefore also investigated the effect of siRNA knockdown of different PAPs on total poly(A) content, in cell proliferation, polyadenylation and effects on gene expression of inflammatory mRNAs.

5.1 The effects of adenosine analogues on polyadenylation

To examine which of the adenosine analogues are polyadenylation inhibitors, investigated the consequences of adenosine analogues on polyadenylation. MCF-7 and NIH3T3 cells were examined for the effects of adenosine analogues on polyadenylation by 3'end labeling. Cells were treated with individual analogues for 2 hours at an indicated concentration. It can be observed from figure 5.1 that 8 amino adenosine showed reduction in length of total poly (A) tail like cordycepin in NIH3T3 cells which was also observed previously in ASM cells (Kondrashov et al; 2012). As observed before, polyadenylation was readily affected by cordycepin (10µM and 50µM). 8 aminoadenosine also showed a reduction but to a lesser extent. Surprisingly, other reputed polyadenylation inhibitors such as 8-chloro adenosine did not affect the total poly(A) length in the cell.

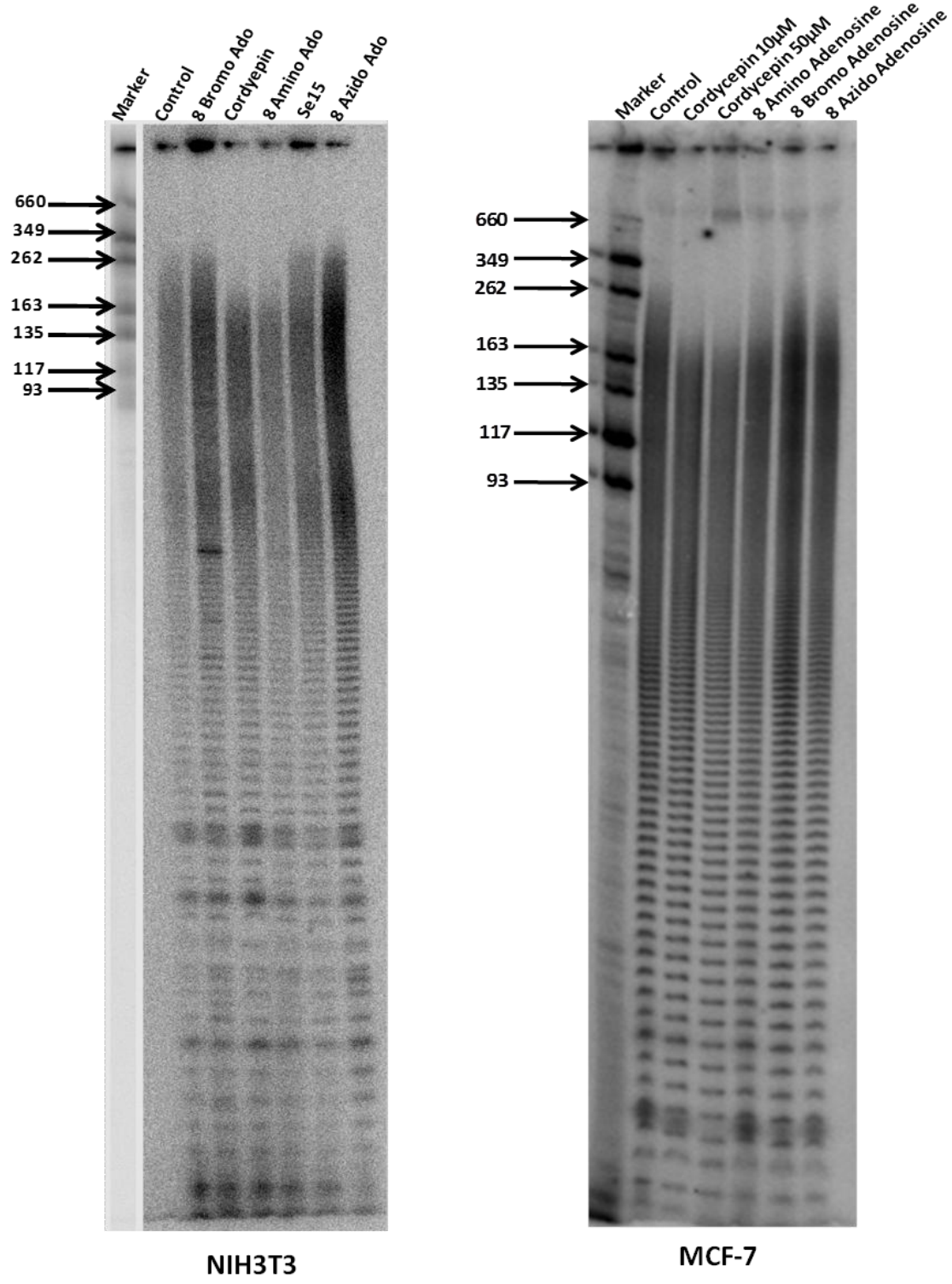


Figure 5.1: The effects of Adenosine analogues at 50µM on total poly (A) tail length:

Indicated cell lines were treated for 2 hours with indicated analogues at 50µM. Total RNA was prepared and labelled at the 3´ end with ^{32}P-pCp along with other necessary components and non-polyadenylated RNA was degraded, which results in a smear which presents the total poly (A) in the cell. The reaction products were analyzed by urea (7M) 15% Polyacrylamide gel electrophoresis. Labelled RNA ladder was used as a marker where each size corresponds to different poly(A) tail lengths in bases.

5.2 Other adenosine analogues as alternative polyadenylation inhibitors

We investigated the effects of the adenosine analogues on the cellular rate of protein synthesis and the levels of P-4EBP in MCF-7 and HELA cells. Both cell lines were treated with indicated adenosine analogues for 2 hours at 50μM and the rate of protein synthesis was determined by S^{35}, while the levels of P-4EBP were determined by ELISA. As shown in figure 5.2. HELA cells show a decline in rate of protein synthesis and in levels of P-4EBP upon treatment with 8-amino adenosine, which has been previously shown to also inhibit polyadenylation by chain termination (Chen et al. 2010). The rate of protein synthesis remains insensitive upon treatment with 8 aminoadenosine in MCF-7 cells but shows greater sensitivity towards levels of P-4EBP, similar to the effects of cordycepin in these cells. 8-amino adenosine also behaves similarly to cordycepin in HeLa cells. All the other adenosine analogues have effects that are distinct from those of cordycepin on the levels of P-4EBP and protein synthesis in HeLa and MCF7 cells, with some affecting protein synthesis in MCF 7 cells (8-azidoadenosine and Se15) and all except Se15 not reducing P-4EBP levels in HeLa cells.Therefore 8-amino adenosine is the only analogue tested that is similar to cordycepin in its effects on protein synthesis and phosphorylation of P-4EBP in different cell lines. The validation of the ELISA method for measuring phospho-4EBP levels by western blotting is necessary, but this could not achieve due to technical reasons.

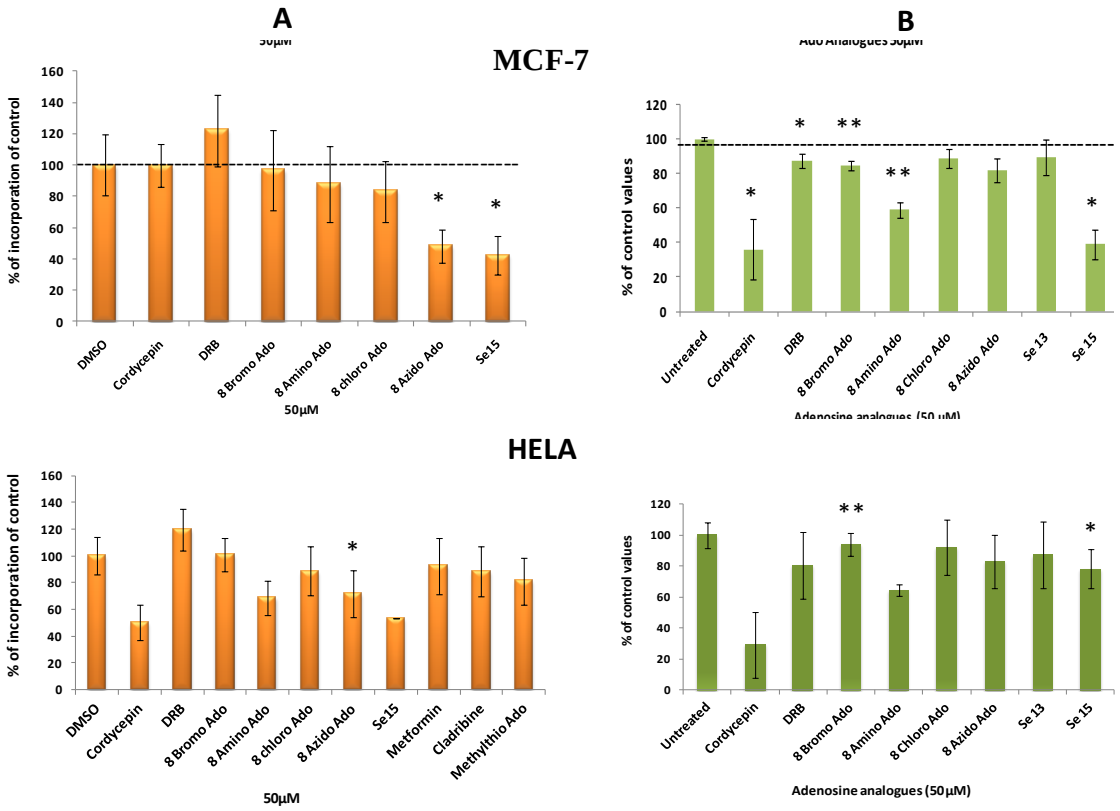


Figure 5.2: The effects of adenosine analogues on MCF-7 and HELA cells:

Indicated cell lines were treated for 2 hours with indicated analogues at 50µM. **A,** Protein synthesis rates were measured by incorporation of ^{35}S labeled amino acids into protein, corrected for total protein concentration and represented as a percent of incorporation of the control. **B,** Levels of P4EBP were determined by in cell ELISA for respective cell lines upon treatment with analogues. Levels of P-4EBP in different cell lines were corrected with total protein concentration. Values are expressed as percentages of the control. The data are shown as the means ±S.D of 4-5 technical replicates for each treatment. Unpaired T-test was performed to show statistical significance compared to untreated control represented as * for P<0.05 and ** for P<0.01.

5.3 The effects of adenosine analogues on cell proliferation

After examining the effects of adenosine analogues on protein synthesis and phosphorylation of P-4EBP, I investigated its effects on cell proliferation in MCF-7 cells. MCF-7 cells were treated for 72 hours with specified compounds and at indicated concentrations and used for cell counting with the trypan blue dye. Cells numbers are represented relative to the seeding density. As shown in figure 5.3 the number of live cells is markedly decreased by cordycepin, 8-azidoadenosine, cladribine and 8-aminoadenosine, while reduction with DRB and 8-bromoadenosine

were also observed. The decline in number of live cells is observed to a much lesser extent after treatment with 8-chloroadenosine, MTA (methylthio ado) and Metformin. Some of the samples that were treated with the adenosine analogues and counted for live and dead cells, were significantly different compared to the untreated samples indicated as * for P<0.05 and ** for P<0.01. This experiment illustrates that many adenosine analogues can affect cell proliferation, but their mechanisms of action are likely to be diverse. Cell proliferation and survival assays are not very useful for elucidating the mechanism of action of a drug.

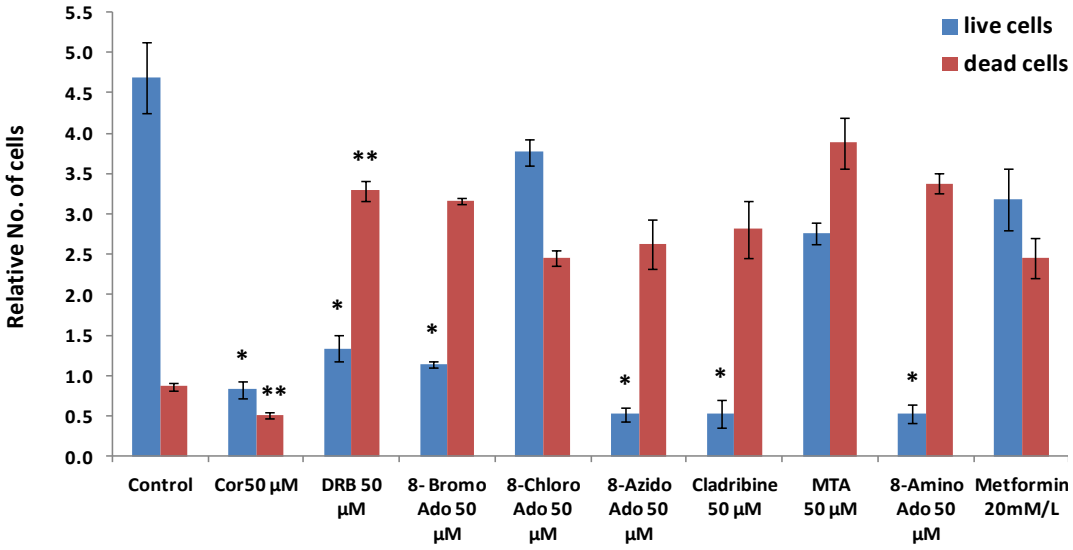


Figure 5.3: The effects on MCF-7 cells by different compounds on cell proliferation:

MCF-7 cells were treated with different compounds and adenosine analogues with indicated concentration for 72 hours and cell counts were done for (dead) blue and (live) white cells using trypan blue dye under the microscope. Results are presented as a relative number of cells. Error bars show standard deviations over 4-5 technical replicates. Statistical significance compared to untreated control was performed by unpaired T-test represented as * for P<0.05 and ** for P<0.01.

5.4 PAP knockdown by SiRNAs

Our data show that cordycepin is certainly a polyadenylation inhibitor in MCF7 cells, but its effects on protein synthesis, 4EBP phosphorylation and cell proliferation could conceivably be mediated by other cellular targets. It is unknown if cordycepin affects the polyadenylation by all PAPs, and what the roles of different PAPs in cell proliferation are. We therefore performed siRNA knockdown experiments. As shown in figure 5.4, knockdown of PAPOLA, PAPOLG and PAPD4 by siRNA

transfection reduced the mRNA levels for each targeted PAP. I then examined the effects of PAP knockdown on cell proliferation. Knockdown of PAPOLA and PAPD4 resulted in a small decline in the number of live cells. The small effect was probably due to the cells reaching confluency early in the experiment, therefore in subsequent experiments lower numbers of cells were seeded (data not shown).

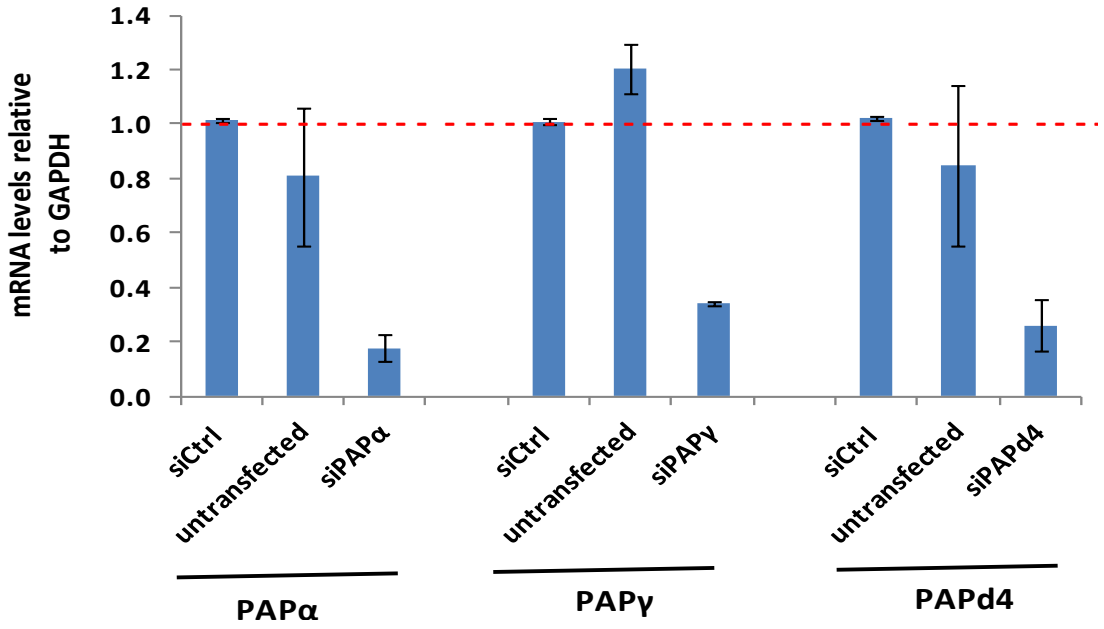


Figure 5.4: PAP knockdown by SiRNAs:

MCF-7 cells were transfected twice with indicated siRNAs for PAPOLA, PAPOLG and PAPD4 as described. mRNA isolation was done after 72 hours of the first transfection at the same time point. A, Levels of spliced mRNA were determined by RT-qPCR and expressed relative to GAPDH mRNA levels. Values for control SiRNA were set at 1. Error bars are standard deviations over two independent biological replicates.

5.5 Combination PAP knockdowns affects cell proliferation

As we observed the effects of individual PAPs knocked down on cell proliferation, we next examined the effects of combination PAP knockdown in MCF-7. Cells were seeded at a density of 0.075 million cells per well and transfected the next day and the day after as described previously and used for cell counting after 72 hours of the first transfection. It can be observed from figure 5.5 that the reduction in cell numbers is similar upon knock down of individual PAPs and this is not increased in

the combination of siPAPOLA with siPAPOLG. However by knocking down different in combinations such as siPAPOLA with siPAPD4, siPAPOLG with siPAPD4 and all three PAPs (siPAPOLA, siPAPOLG, siPAPD4), a much greater decline in cell numbers is observed. As shown in the figure, most of the samples for live cells were statistical significant indicated as * for P<0.05, ** for P<0.01 and *** for P<0.001. These data clearly indicate that all three poly(A) polymerases we examined are required for cell proliferation. As we saw effects on cell proliferation we next examined the changes in total poly (A) tail length by knocking down either of these PAPs.

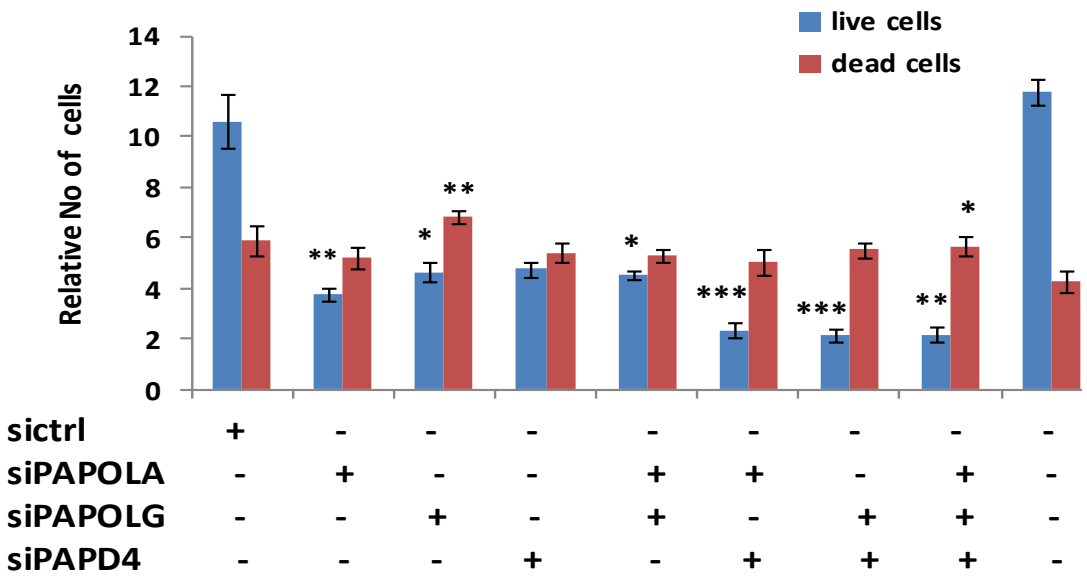


Figure 5.5: Cell proliferation is affected by combination PAP knockdowns in MCF-7 cells:

Cells were transfected twice in combination with indicated siPAPs for PAPOLA, PAPOLG and PAPD4 as described. Cell counts were done after 72 hours of first transfection by counting (dead) blue and (live) white cells using trypan blue dye under microscope. Results are presented as relative numbers of cells and measurements were independent biological triplicates expressed as the mean ±S.D. Statistical significance compared to control (No cordycepin) were determined by Paired T-test indicated as * for P<0.05, ** for P<0.01 and *** for P<0.001.

5.6 The effects of PAP knockdowns on polyadenylation

To examine the effects on polyadenylation on the knock down of individual PAPs we performed another 3’ end labeling experiment. The cells were transfected as described previously followed by total RNA isolation. RNA was labeled and processed for degradation of non-polyadenylated RNA by RNase T1 and RNase A and electrophoresis on urea polyacrylamide gel. As shown in figure 5.6 the length of total poly(A) tail was not affected by the knocking down of any indicated PAPs. It indicates that bulk polyadenylation is not dependent on any of the individual PAPs we tested and that there is probably some redundancy in the function of different PAPs.


Marker
untransfected
sictrl
sialpha
sigamma
sigld2
349
262
163
135
117
93

Figure 5.6: The effects of PAP knockdowns on total poly (A) tail length in MCF-7:

MCF-7 cells were transfected twice with indicated siPAPs for PAPOLA, PAPOLG and PAPD4 within 72 hours. Total RNA was prepared and labelled at the 3´ end with ^{32}P-pCp along with other necessary components and non-polyadenylated RNA degraded which results in a smear presenting the total poly(A) in the cell. The reaction products were analyzed by urea (7M) 15% Polyacrylamide gel electrophoresis. Labelled RNA ladder was used as a marker where each size corresponds to different poly(A) tail lengths in bases.

5.7 The effects of PAP knock down on the level of selected mRNAs

We determined the effects on polyadenylation on gene expression of inflammatory and cordycepin potentially targeted mRNAs by siPAP knocked down. MCF-7 cells were transfected as described previously followed by total RNA isolation. RT-qPCR was performed on each mRNA and values were expressed relative to untreated (sictrl), which was set at 1. GAPDH was used as a control for normalization. Unexpectedly, siRNA transfection induced the inflammatory mRNAs. We found that this was due to the Interferin transfection reagent, as this effect occurred even in the absence of any siRNAs (data not shown). We therefore compared the mRNA levels only to the control siRNA treated samples. As shown in figure 5.7 not much effect was observed on CXCL1 expression, while for IL8 lower levels were observed in PAPD4 knockdown only. No effects were observed on expression of TWIST1 and ACTB mRNAs. So, in contrast to in ASM cells (Kondrashov et al; 2012), the effects of PAP knockdown did not mimic that of cordycepin in MCF7 cells.

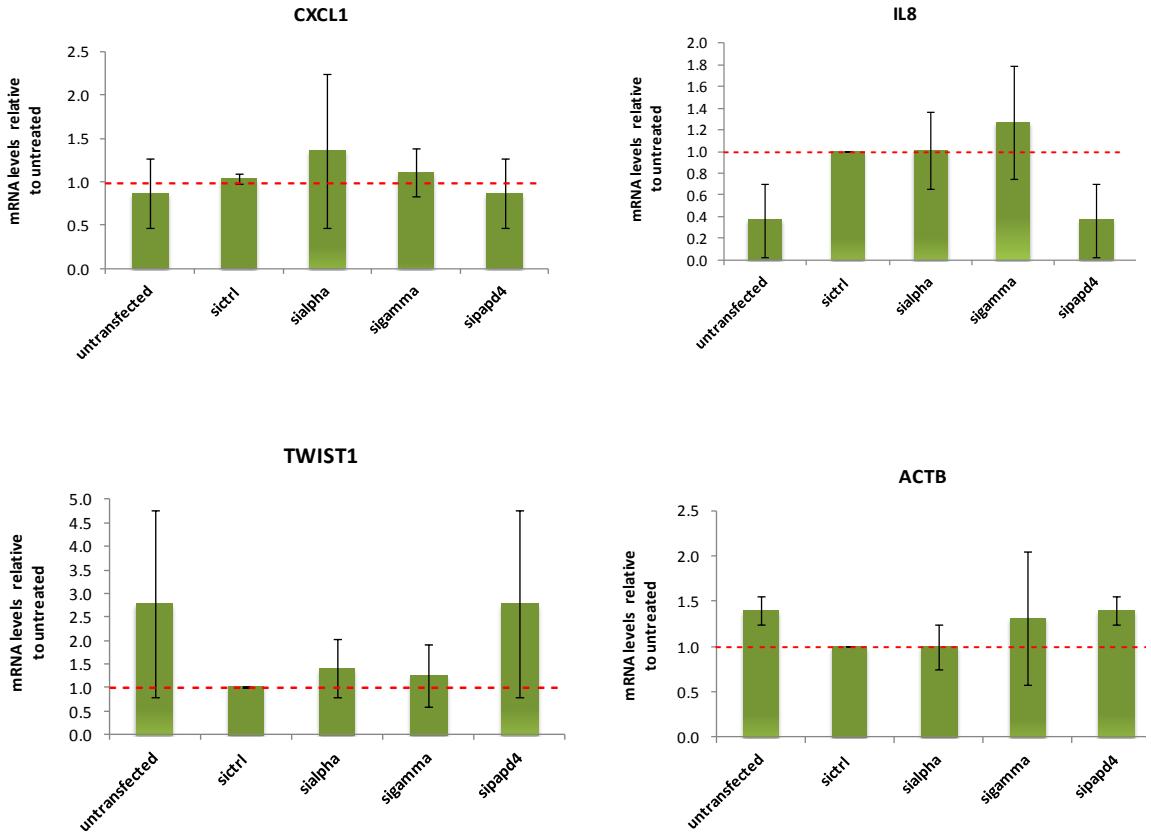


Figure 5.7: The effects of PAP knockdown on MCF-7 cells:

MCF-7 cells were transfected twice with indicated siPAP for PAPOLA, PAPOLG and PAPD4 within 72 hours as described. Total RNA was isolated and RT-qPCR was performed as described. For a control housekeeping mRNAs B-actin (ACTB) and RPL10A were used. The mRNA levels are expressed relative to GAPDH mRNA and untreated values for control siRNA were set at 1. Error bars are standard deviations over three independent biological replicates. Statistical significance compared to sicontrol was determined by Paired T-test but none of them found to be statistically different from the sicontrol.

In these experiments, knockdown of poly(A) polymerases does not appear to have similar cellular effects to cordycepin. It is possible that this is due to the longer duration of knockdown compared to drug treatment or to the redundant functions of the poly(A) polymerases. It may also be because the effect of cordycepin is not similar to the removal of an RNA polymerase, but has dominant effects through its stabilization of the cleavage complex. An alternative approach could be using dominant negative mutants of the relevant PAPs to study the effects of polyadenylation on levels of cordycepin sensitive genes.

5.8 Effects of adenosine analogues on inflammatory mRNAs

We next evaluated the effects of adenosine analogues on levels of inflammatory mRNAs in MCF-7 cells. Cells were treated with adenosine analogues at 50µM for 2 hours followed by total RNA isolation. RT-qPCR was done on inflammatory mRNAs normalized to GAPDH and values for untreated were set as 1. It can be observed from figure 5.8 that CXCL1 is sensitive to both cordycepin 50µM and 8-aminoadenosine treatments. However 8 azidoadenosine also shows a small reduction in levels of CXCL1. Cordycepin at both dosages, 8 aminoadenosine and 8 azido adenosine all reduced the levels of IL8 mRNA. The RPL10A and ACTB housekeeping mRNAs remained unchanged. We previously observed the reduction in induction of inflammatory mRNAs such as CXCL1 and IL8 by cordycepin and 8 amino adenosine treatments in ASM cells (Kondrashov et al; 2012). Therefore, it can be concluded that 8 amino adenosine is similar to cordycepin in its effects on polyadenylation, protein synthesis, 4EBP phosphorylation and gene expression in MCF-7 cells.

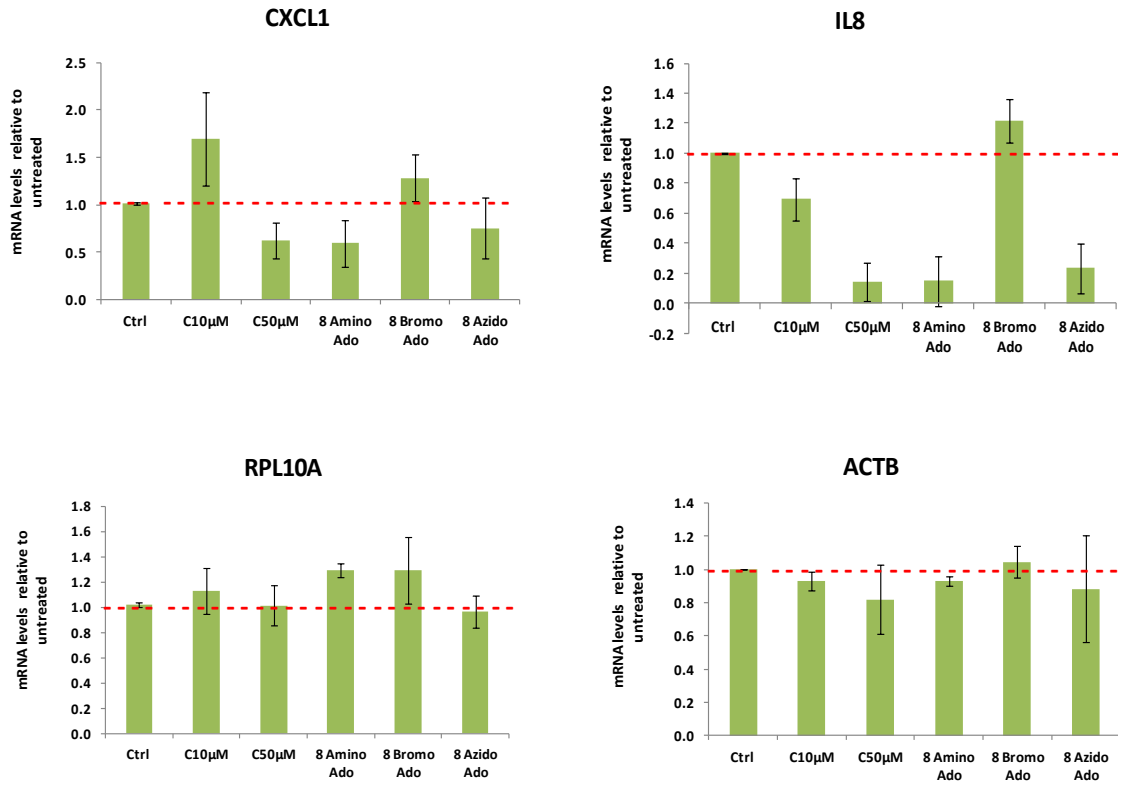


Figure 5.8: Adenosine analogues inhibit expression of inflammatory mRNAs in MCF-7:

MCF-7 cells were treated with adenosine analogues (50μM) for 2 hours. Total RNA was isolated and RT-qPCR was performed as described. Housekeeping mRNAs encoding β-actin (ACTB) and a ribosomal protein (RPL10A) were used as a control. The mRNA levels are expressed relative to GAPDH mRNA and untreated control values (no cordycepin) were set at 1. Experiments were independent biological triplicates and data are expressed as means ±S.D. Statistical significance compared to control was determined by Paired T-test but none of the examined mRNAs were found to be sensitive.

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5.9 Effects on cordycepin sensitive mRNAs are post-transcriptional

In order to examine whether cordycepin affects mRNA synthesis or stability, we evaluated the effects of cordycepin on nuclear and cytoplasmic fractions. Cells were treated for 2 hours with cordycepin followed by separation of nuclear and cytoplasmic fractions. RT-qPCR was done on each fraction and normalized to GAPDH. As shown in figure 5.9A, the levels of CXCL1, IL8 and TWIST1 mRNA were markedly reduced in cytoplasmic fractions by cordycepin, while less effect was observed in the nuclear fraction, indicating that effects of cordycepin were post-transcriptional in MCF-7 cells. The data suggest that cordycepin induces degradation of sensitive mRNAs soon after they are exported from the nucleus. In figure 5.9 B, the levels of unspliced pre-mRNAs in nuclear RNA were determined. Un-CXCL1 shows a small reduction in mRNA levels, while un-IL8 remains unchanged. We previously demonstrated that cordycepin increases the levels of unspliced pre-mRNA for CXCL1 compared to CCL2, which remain unchanged. Meanwhile, levels of unspliced pre-mRNA of IL8 were reduced in ASM cells (Kondrashov et al; 2012). Nascent and mature RPL10A mRNA levels were used as controls, which remain unchanged by cordycepin treatment. Figure 5.9 C, shows the ratio of unspliced over spliced RPL10A for each sample in the nuclear and cytoplasmic fractions, demonstrating successful fractionation. All experiments were performed in three independent biological replicates and statistical significance was calculated by a paired T test compared to untreated controls for each mRNA.

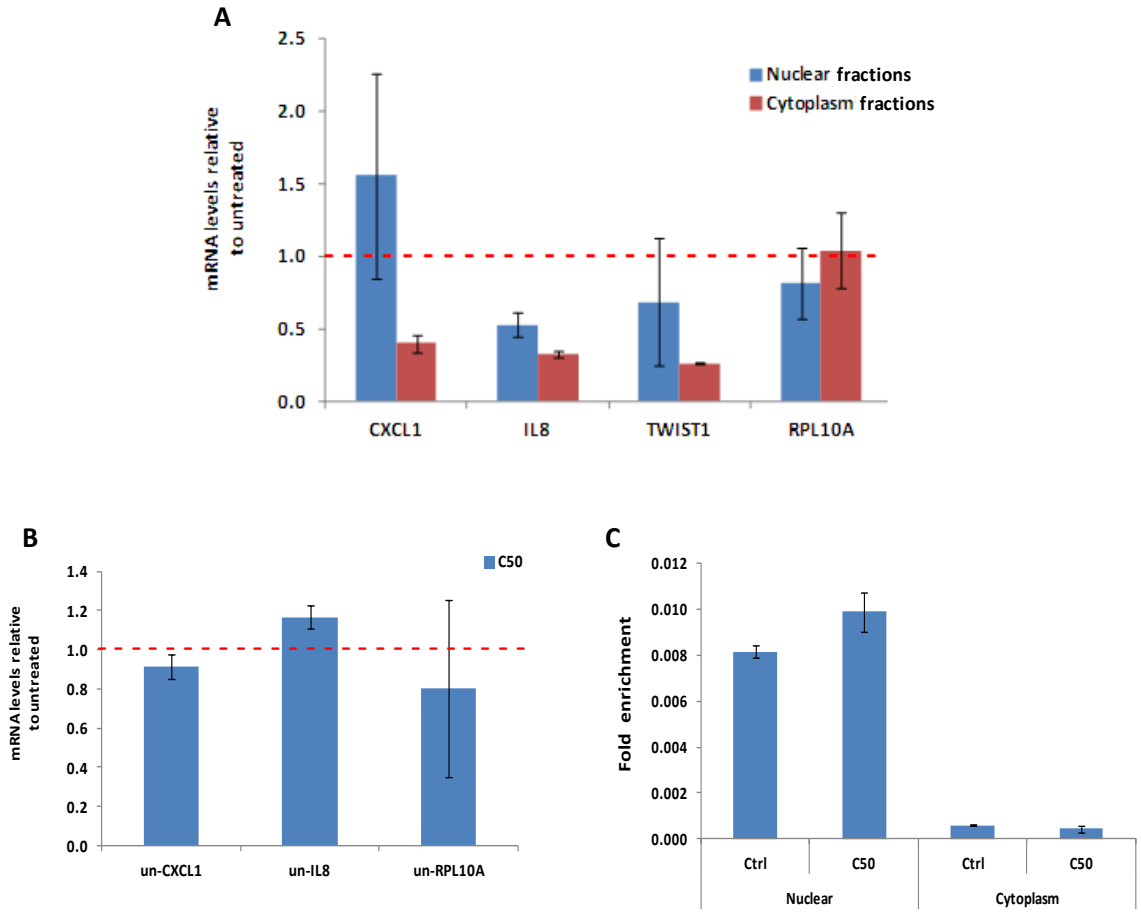


Figure 5.9: Nascent and mature inflammatory mRNAs affected by cordycepin:

MCF-7 cells were treated with cordycepin (50µM) for 2 hours and nuclear and cytoplasmic fractions were separated as described. **A,** Total RNA was isolated and RT-qPCR for mature mRNA (spliced) was performed on nuclear and cytoplasmic fractions. The mRNA levels were expressed relative to GAPDH mRNA and control values of untreated (no cordycepin) were set at 1. Error bars represent standard deviations over independent biological triplicates. **B**, RT-qPCR for unspliced pre-mRNA was performed on the nuclear fraction. The mRNA levels are expressed relative to GAPDH mRNA and control values of untreated (no cordycepin) were set at 1. Error bars represent standard deviations over independent biological triplicates. **C,** RNA fractions were separated followed by RT-qPCR for spliced and unspliced RPL10A mRNA. The ratio of unspliced over spliced for each sample was divided by this ratio in the nuclear and cytoplasmic fraction. Error bars are standard deviations over independent biological triplicates. Statistical significance compared to control (No cordycepin) was determined by a paired T-test for A, B and C, but none of them were found to be statistically different from the control.

5.10 Investigation of the cordycepin sensitivity of mRNAs in cancer and mTOR related pathways

It is known that cordycepin affects mTOR signaling and components of the upstream PI3K pathway have been reported to be subject to regulation by the cytoplasmic polyadenylation element binding protein (Wong et al. 2010). We therefore decided to investigate the mRNAs involved in mTOR signalling and other candidates for regulation by CPEB like TWIST1 (Nairismagi et al. 2012) in an attempt to identify more target mRNAs for comparison of cordycepin with other treatments in MCF-7 cells. Cells were treated with cordycepin 50μM for 2 hours followed by total RNA isolation. RT-qPCR was performed normalized to GAPDH and untreated values were set at 1. The key players of mTOR pathway including cancer related and control mRNAs were investigated as shown in figure 5.10 as separated by a space. None of the tested mRNAs were sensitive to cordycepin except TWIST1 and to a lesser extent PIK3CB mRNA. Housekeeping mRNAs ACTB and RPL10A remain unchanged. Experiments were done in three independent biological replicates and none of the examined mRNAs were found to be significantly different from the untreated by paired T-test.

We also examined the cordycepin sensitivity of several mRNAs that were linked to the machinery of polyadenylation complexes. This was on the argument that there might be autoregulation, however we were unable to find any other sensitive mRNAs. Therefore in order to find cordycepin targeted mRNAs there is a need to create high throughput data in order to identify more target mRNAs of cordycepin, such as by microarray analysis.

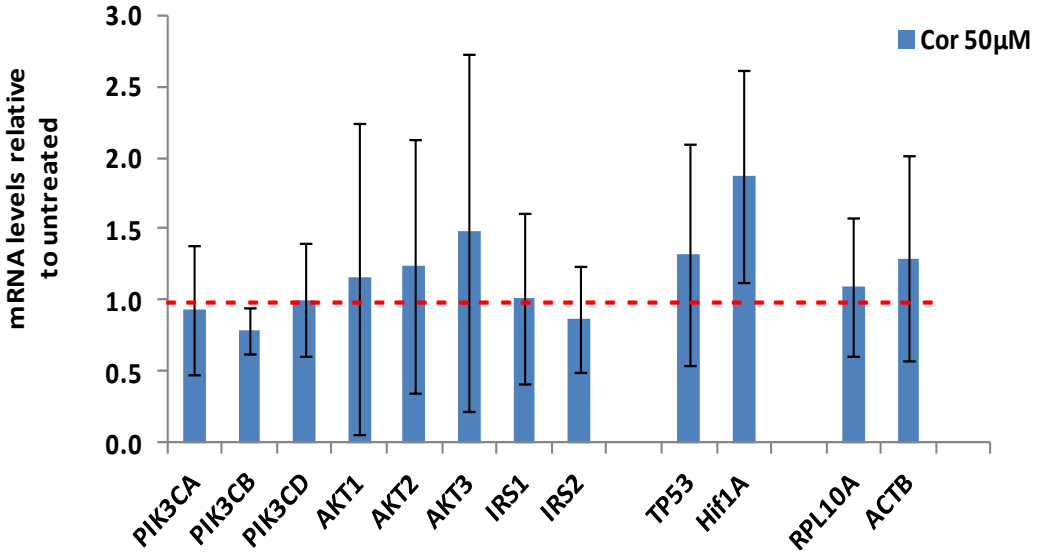


Figure 5.10: Cordycepin does not affect expression levels of mTOR and cancer mRNAs:

Total RNA was isolated from MCF-7 cells treated with cordycepin (50µM) for 2 hours. RT-qPCR was performed and the mRNA levels were expressed relative to GAPDH mRNA. Control values of untreated (no cordycepin) were set at 1. Error bars represent standard deviations of independent biological triplicates. Statistical significance compared to control (No cordycepin) was determined by paired T-test but none of the examined mRNAs were found to be significant.

5.11 Discussion

The half life of the cordycepin is very short due to its degradation in the blood by an enzyme called adenosine deaminase. To counteract this effect, cordycepin is usually administered in combination with the adenosine deaminases inhibitor deoxycoformycin or pentostatin, however there is some limitation in using these drugs due to their toxicity. To design more stable cordycepin analogues, we first need to firmly establish its mechanism of action. If cordycepin works by inhibiting polyadenylation, which PAPs is it targeting? Therefore other adenosine analogues were investigated which could be stable and could have different specificity for individual PAPs. Like cordycepin, other adenosine analogues also show different activities towards various cell lines. 8 amino adenosine, 8 azido adenosine and Se15 inhibit cellular rates of protein synthesis, perhaps by reducing the phosphorylation of 4EBP. In MCF-7 cells Se15 reduces the rate of protein synthesis by exerting its

effects on P-4EBP. In MCF-7 cells cell proliferation was found to be sensitive to 8 aminoadenosine, 8 azidoadenosine and cladribine. Therefore, among all tested analogues 8 azidoadenosine may reduce cell proliferation by affecting the rate of protein synthesis and phosphorylation of 4EBP.

We also evaluated the effects on cell proliferation of poly(A) polymerase (PAP) alpha, gamma and Papd4 knockdowns and found all PAPs are necessary for cell proliferation. However no significant difference has been observed by PAP knock downs on total polyadenylation and no changes in levels of gene expression were observed. However we previously published that knockdown of PAPOLA reduces inflammatory response in ASM cells. We also investigated of the level at which cordycepin affects mRNA abundance and we observed post-transcriptional effects on all mRNAs we examined (CXCL1, IL8 and TWIST1). To elucidate the cordycepin mode of action in MCF7 cells in detail we screened several mRNAs in order to find cordycepin sensitive mRNAs but we only found 2 mRNAs among all examined. We therefore proceeded to conduct a microarray in order to find more cordycepin targetted mRNAs.

We therefore conclude that 8 aminoadenosine is the only compound that has similar cellular effects to cordycepin (protein synthesis, 4EBP level, proliferation and gene expression), as well as the only other compound that affects total poly(A) tail size. This indicates that the polyadenylation inhibition and the cellular effects are causally linked. Knockdown of 3 poly(A) polymerases in MCF-7 cell lines indicates that all are required for cell proliferation but that it does not have effects similar to cordycepin on gene expression. As both cordycepin and 8 aminoadenosine have been reported to act as chain terminators, it is possible that their effect is more due to a dominant negative effect mediated by an arrested complex. This would possibly be better mimicked by mutant PAPs, rather than by knockdown. Alternatively, there may be more redundancy in the PAPs in MCF7 cells, and multiple knockdowns should be tried. A genome wide study needs to be done to identify potential candidate mRNAs targeted by cordycepin.

6 Identifying cordycepin sensitive mRNAs in MCF7 cells

In this chapter we attempt to elucidate the mechanism of action of cordycepin in more detail by performing microarray analysis, which allows the study of the expression of thousand of genes simultaneously in one sample. This experiment was done in collaboration with Professor Anne Willis (MRC Toxicology unit, Leicester) on MCF-7 cells treated with cordycepin (50µM) for 2 hours in a four independent biological replicates. Enrichment of genes in functional groups was also investigated using the DAVID (Database for Annotation, Visualisation and Integrated Discovery). The lists of significantly expressed genes were sorted according to fold change and the P-value for the significance of the change. Some significantly changed candidates were selected for validation by RT-qPCR. We selected four cordycepin targeted mRNAs and examined the effects of adenosine analogues and PAP knockdowns. To elucidate the mode of action of cordycepin on these target mRNAs in detail we also investigate the stage in mRNA processing that is affected by cordycepin.

6.1 Functional clustering of significant genes in microarray analysis

The availability of different data analysis software makes it possible to analyze the data generated by high throughput genome wide screening to cluster the genes according to functional annotation on a wide range of criteria. Such types of tools are helpful in categorizing the genes in order to identify particular cellular functions affected by cordycepin. The list generated from microarray data analysis were categorized into two groups, firstly sorted according to a real fold change greater than 1.66 (up regulated by cordycepin) and with fold change less than 0.66 (down regulated by cordycepin), as calculated by Agilent software. Both these groups were filtered to only include $P< 0.01$ as shown in table 9 and table 10.

Table 9: List of significantly up regulated genes:

Data of microarray analysis were sorted on the basis of fold change (FC > 1.66) with P-values (P< 0.01).

Gene	P.Value	RealFC	Gene	P.Value	RealFC
LOC100652740	0.000	12.820	XLOC_008698	0.010	2.347
LOC646719	0.001	6.306	CHRNA10	0.008	2.347
LOC100507018	0.007	5.869	ENST00000450112	0.008	2.342
FAM90A7	0.000	5.575	GLB1L	0.002	2.337
AK025118	0.001	5.476	PTPRK	0.008	2.337
MGC11082	0.001	4.904	C16orf55	0.003	2.304
LRRC66	0.000	4.861	ENST00000535853	0.004	2.301
FAM83E	0.002	4.734	XLOC_000377	0.004	2.294
LOC100506262	0.000	4.667	SART1	0.006	2.273
ZNF865	0.001	4.658	LOC340335	0.007	2.271
A_33_P3223860	0.001	4.578	CRTC1	0.003	2.256
ARPC4-TTLL3	0.000	4.490	THC2565581	0.005	2.252
KIAA1875	0.003	4.463	FHL2	0.008	2.244
PRPF40A	0.001	4.447	LOC100128563	0.007	2.243
DENR	0.003	4.441	ARL9	0.002	2.240
TMED6	0.002	4.310	APOE	0.004	2.235
LOC100132006	0.000	4.184	ENST00000518331	0.007	2.217
CDRT1	0.001	4.115	XLOC_l2_001091	0.005	2.206
XLOC_014513	0.007	4.115	LOC100147773	0.008	2.204
FOXD4	0.005	4.074	CENPN	0.009	2.202
XLOC_000153	0.003	3.944	FOXP1-IT1	0.006	2.192
XLOC_000152	0.000	3.936	LOC100132356	0.006	2.189
KCNJ14	0.001	3.921	HCG18	0.008	2.174
AMY1C	0.005	3.910	PNPLA7	0.004	2.166
THC2767054	0.004	3.857	TAL2	0.004	2.136
DQ272581	0.002	3.854	PRRT2	0.004	2.122
ENST00000326348	0.002	3.818	XLOC_008828	0.003	2.122
C1orf162	0.002	3.736	RASL10A	0.003	2.118
FBXW10	0.003	3.733	A_33_P3381305	0.010	2.107
A_24_P203814	0.001	3.663	LOC285178	0.008	2.088
XLOC_003522	0.003	3.642	SPRED3	0.003	2.087
C20orf106	0.002	3.634	MCART1	0.007	2.083
FAM90A1	0.001	3.630	THC2725851	0.003	2.079
FLJ38717	0.001	3.573	LOC100130285	0.003	2.077
PP12719	0.002	3.517	XLOC_l2_013873	0.007	2.077
FES	0.001	3.516	XLOC_l2_015213	0.008	2.065
XLOC_007942	0.001	3.475	LOC100499221	0.005	2.058
SCARNA15	0.006	3.432	FLJ31662	0.008	2.057
ENST00000355500	0.003	3.396	LOC100131825	0.005	2.055
P39195	0.002	3.369	AK074630	0.004	2.054
XLOC_l2_008221	0.005	3.321	C21orf58	0.006	2.046
XLOC_002517	0.003	3.310	FLJ42627	0.006	2.039

XLOC_l2_013873	0.004	3.278	ZNF445	0.006	2.019
LOC96610	0.001	3.276	TAS2R30	0.010	2.006
AOC3	0.001	3.247	FLJ32224	0.010	1.988
XLOC_004924	0.001	3.199	LOC644450	0.006	1.987
XLOC_005244	0.010	3.193	AY129018	0.006	1.974
RBM26-AS1	0.003	3.180	LOC143286	0.005	1.971
XLOC_l2_013001	0.003	3.107	LOC100132356	0.005	1.967
XLOC_012895	0.009	3.048	FAT3	0.005	1.964
XLOC_012667	0.001	3.042	CARD9	0.006	1.963
THC2539584	0.003	3.029	LOC100130000	0.009	1.963
ALPK3	0.004	3.024	XLOC_014226	0.006	1.957
XLOC_l2_010558	0.001	2.983	PIGL	0.009	1.954
XLOC_001788	0.002	2.954	XLOC_012359	0.005	1.952
XLOC_l2_009273	0.004	2.886	XLOC_l2_013846	0.010	1.942
XLOC_l2_010558	0.003	2.878	ZSCAN12P1	0.008	1.935
XLOC_004231	0.010	2.860	AK022609	0.007	1.935
LOC100131792	0.006	2.849	XLOC_l2_011043	0.007	1.933
LOC100216546	0.005	2.824	LOC203274	0.005	1.923
KIAA1683	0.002	2.823	ENST00000411845	0.008	1.917
LOC283693	0.003	2.822	WNK4	0.007	1.916
MASP2	0.001	2.813	GALK1	0.008	1.914
XLOC_009507	0.007	2.804	SNORD35B	0.007	1.906
DNAJC30	0.009	2.786	PCDHGA2	0.009	1.902
LOC100130887	0.001	2.759	CYTH1	0.008	1.902
LOC100507410	0.010	2.756	P39194	0.009	1.877
LOC145757	0.002	2.737	A_33_P3280502	0.007	1.869
XLOC_l2_009273	0.001	2.735	LOC100505717	0.006	1.858
LOC399900	0.003	2.723	LOC728537	0.008	1.857
A_24_P170095	0.002	2.708	LOC100505648	0.010	1.855
XLOC_002313	0.006	2.686	ENST00000439955	0.010	1.854
HPS1	0.007	2.671	AF264629	0.008	1.854
ENST00000378337	0.001	2.667	MACROD2	0.008	1.844
HOXB9	0.006	2.627	FLJ37453	0.008	1.842
XLOC_l2_012847	0.007	2.624	LOC100128811	0.008	1.841
LEPROTL1	0.001	2.622	WDR78	0.007	1.837
LOC100289230	0.009	2.621	LAG3	0.008	1.832
XLOC_002996	0.001	2.603	LOC401320	0.008	1.827
LOC100507501	0.004	2.599	SLC25A16	0.008	1.800
DQ786257	0.002	2.595	FRMD4A	0.008	1.790
XLOC_001942	0.005	2.571	LOC645261	0.010	1.782
THC2674845	0.010	2.565	ENST00000367690	0.009	1.780
XLOC_003405	0.002	2.559	C17orf76-AS1	0.009	1.780
PAFAH1B2	0.004	2.558	TTC9C	0.009	1.778
XLOC_002997	0.005	2.556	ENST00000354995	0.010	1.748
KLRAP1	0.010	2.555	AK131313	0.010	1.746
XLOC_002779	0.010	2.547	LOC338799	0.009	1.741
FLJ45482	0.002	2.545	LOC100129387	0.010	1.733

BLZF1	0.009	2.542	ENST00000354995	0.010	1.748
ZADH2	0.006	2.499			
XLOC_006973	0.002	2.477			
FLJ11710	0.006	2.473			
AVIL	0.003	2.469			
ENST00000535190	0.010	2.469			
XLOC_011248	0.005	2.448			
XLOC_011837	0.003	2.441			
ERVMER34-1	0.002	2.427			
THC2569478	0.004	2.415			
LOC100132352	0.004	2.393			
XLOC_l2_015038	0.002	2.373			
XLOC_l2_006578	0.010	2.358			
LOC338620	0.009	2.349			

Table 10: List of significantly down regulated genes:

Data of microarray analysis were sorted on the basis of fold change (FC < 0.66) with values (P< 0.01).

Gene	P.Value	RealFC	Gene	P.Value	RealFC
SNAR-A3	0.000	0.069	SPATA2L	0.002	0.412
CYR61	0.001	0.077	MIR143HG	0.007	0.413
SNAR-D	0.001	0.084	SNHG7	0.002	0.413
SNAR-B2	0.001	0.084	HERPUD1	0.003	0.414
SNAR-H	0.001	0.088	C7orf74	0.002	0.414
SNAR-G2	0.001	0.095	GRPEL2	0.003	0.415
CHAC1	0.000	0.098	SPSB1	0.005	0.416
PLK3	0.000	0.108	NFKBIA	0.010	0.417
VTRNA1-3	0.007	0.112	JUNB	0.003	0.417
SNAR-F	0.001	0.115	ZNF283	0.006	0.417
NAB2	0.001	0.117	PLEKHO2	0.007	0.419
MYC	0.000	0.131	ZNF263	0.008	0.421
TIPARP	0.001	0.133	ZNF8	0.004	0.421
C15orf39	0.000	0.144	FOXL2	0.004	0.423
PPP1R3C	0.007	0.170	EGR1	0.003	0.424
MIRLET7BHG	0.001	0.178	GEM	0.009	0.424
CITED2	0.001	0.191	SIK1	0.004	0.425
SNAR-G1	0.001	0.192	STK40	0.004	0.428
ID1	0.003	0.192	RNF216P1	0.004	0.429
ERRFI1	0.002	0.194	SNORA70	0.010	0.431
SERTAD1	0.001	0.195	FOXA1	0.004	0.432
SLC25A25	0.002	0.196	C15orf42	0.003	0.434
SGK1	0.001	0.197	MALAT1	0.007	0.434
DUSP2	0.001	0.198	ZNF668	0.002	0.436
ZNF134	0.009	0.204	CXCR4	0.010	0.437
ADM	0.002	0.205	AEN	0.009	0.437
ADRB2	0.002	0.207	HJURP	0.007	0.438
TGIF1	0.001	0.210	C6orf145	0.007	0.438
DDIT4	0.003	0.213	GPATCH3	0.002	0.438
DLX2	0.003	0.213	NXT1	0.005	0.439

ARID5B	0.005	0.214	SNORA70E	0.009	0.439
OBFC2A	0.000	0.217	ZNF165	0.009	0.440
CTGF	0.005	0.217	RAPGEF2	0.002	0.441
ID3	0.002	0.220	SNORA70G	0.008	0.442
RND3	0.001	0.220	ZBTB45	0.003	0.442
C3orf19	0.004	0.224	CYP1A1	0.004	0.443
TRIB1	0.002	0.228	EFNA1	0.006	0.443
WEE1	0.002	0.228	C8orf4	0.009	0.444
MED9	0.004	0.229	ZNF629	0.002	0.444
ATF3	0.002	0.237	PELO	0.004	0.446
HILPDA	0.001	0.238	ZNF689	0.004	0.447
DOK7	0.002	0.241	IL11	0.006	0.448
DUSP1	0.001	0.243	SEMA4C	0.007	0.449
CDC42EP2	0.001	0.246	PPRC1	0.003	0.449
KCNK5	0.004	0.246	XKR8	0.007	0.450
MAP3K14	0.001	0.246	SLC38A2	0.003	0.451
KLHL21	0.001	0.247	CDR2	0.003	0.451
SESN2	0.002	0.249	XLOC_006513	0.003	0.452
ZBTB3	0.001	0.250	LRRC14	0.002	0.453
PHF13	0.005	0.253	SCARNA9	0.009	0.454
CSRNP1	0.001	0.257	ZNF646	0.004	0.454
FOSL1	0.001	0.265	ATF3	0.005	0.460
LIF	0.002	0.266	MID1IP1	0.007	0.460
TGIF1	0.007	0.266	PLEKHG3	0.006	0.461
LCMT2	0.002	0.267	RUSC2	0.004	0.461
JUN	0.001	0.269	ZNF317	0.005	0.462
BRF2	0.003	0.271	ZNF584	0.003	0.462
DNAJB9	0.006	0.271	ZNF628	0.003	0.463
F3	0.002	0.271	TICAM1	0.007	0.463
MAD2L1BP	0.001	0.273	RPP38	0.006	0.464
IER3	0.002	0.273	PHF23	0.003	0.465
EIF1AD	0.001	0.275	SNORA70C	0.010	0.466
IER3	0.001	0.277	NUAK1	0.004	0.466
KTI12	0.010	0.278	PHF23	0.004	0.466
FAM46A	0.005	0.278	FAM125B	0.008	0.466
SOCS3	0.002	0.278	ND2	0.004	0.466
AMOTL2	0.010	0.279	ZNF511	0.008	0.466
FBXW7	0.003	0.279	NFIL3	0.007	0.467
CLCF1	0.001	0.281	PLEKHF1	0.006	0.468
RUNX1	0.002	0.282	ARMC7	0.004	0.469
ZNF830	0.005	0.282	ZNF394	0.010	0.472
DDX28	0.001	0.284	MIRLET7BHG	0.008	0.474
NUAK2	0.003	0.284	EPHA2	0.008	0.475
ITPRIP	0.001	0.287	NR4A3	0.009	0.475
SNORA61	0.005	0.289	ZBTB5	0.008	0.475
IER5	0.001	0.290	C5orf30	0.007	0.479
PLK2	0.001	0.291	TLE4	0.003	0.479
HPS6	0.003	0.293	KLF6	0.006	0.481
PLAGL2	0.001	0.295	ZBED5	0.007	0.481
ABL2	0.001	0.295	CSTF1	0.008	0.484
TRIM13	0.004	0.300	BRD2	0.010	0.487
C17orf51	0.002	0.300	DYRK1A	0.010	0.488
KLHL25	0.001	0.305	ZNF394	0.010	0.488
RHEBL1	0.004	0.306	RG9MTD1	0.007	0.488

PPP1R15A	0.001	0.307	FAM46A	0.005	0.489
ZFP36	0.002	0.307	BTG1	0.010	0.490
RHOB	0.007	0.311	AEN	0.010	0.490
ZNF48	0.002	0.312	SIAH1	0.007	0.492
BCAR3	0.001	0.313	NRG1	0.006	0.493
CSRNP1	0.002	0.317	PILRB	0.005	0.493
PPM1D	0.009	0.318	ZNF7	0.007	0.495
TOB2	0.007	0.320	SIAH2	0.007	0.495
BCDIN3D	0.003	0.321	PFKFB3	0.007	0.495
KLF5	0.006	0.322	C18orf21	0.005	0.496
C17orf51	0.001	0.322	C19orf48	0.006	0.497
KLHL26	0.007	0.323	MLL5	0.010	0.497
SNHG12	0.002	0.324	ETAA1	0.008	0.499
TUFT1	0.009	0.325	ARL4A	0.005	0.500
HBEGF	0.002	0.329	FAM83G	0.005	0.503
C17orf51	0.001	0.332	PPP1R3B	0.005	0.506
RELT	0.001	0.336	PABPC4L	0.007	0.506
BAMBI	0.004	0.337	TRIM27	0.005	0.507
FRAT2	0.007	0.339	POLR1C	0.005	0.509
FOXQ1	0.002	0.342	FZD5	0.009	0.509
E2F6	0.003	0.343	KIAA1826	0.005	0.510
ING1	0.001	0.344	FBXO34	0.010	0.511
C17orf51	0.001	0.344	FBXL5	0.008	0.511
LDLR	0.002	0.348	MEX3C	0.008	0.512
TIGD5	0.001	0.350	AIFM2	0.005	0.512
TBCC	0.002	0.351	CDT1	0.007	0.513
CBX8	0.003	0.352	SNX11	0.007	0.514
ST3GAL1	0.002	0.352	ZKSCAN2	0.005	0.515
ZNF574	0.002	0.353	PHLPP2	0.006	0.516
SERTAD2	0.002	0.358	SNORD79	0.009	0.516
MIER3	0.003	0.359	MYNN	0.005	0.516
IER2	0.004	0.359	PGBD3	0.007	0.518
PIM3	0.002	0.360	RASSF1	0.006	0.519
C3orf52	0.001	0.361	ZNF581	0.009	0.520
PLD6	0.002	0.361	GET4	0.008	0.520
PTGER4	0.007	0.362	USP42	0.007	0.520
ZNF134	0.001	0.363	HSF2	0.010	0.521
SNAI1	0.003	0.364	GLIS2	0.010	0.521
SPRY2	0.005	0.364	ZNF189	0.005	0.521
TRIM11	0.004	0.367	CD86	0.008	0.521
PPP1R15B	0.004	0.370	RARA	0.007	0.522
CHAMP1	0.007	0.370	SMYD4	0.006	0.527
RPUSD2	0.005	0.371	ADAT3	0.006	0.527
MESDC1	0.002	0.371	MAFG	0.007	0.528
MAFF	0.004	0.372	DHX35	0.009	0.530
ZNF784	0.002	0.372	IL6	0.008	0.531
SMAD6	0.001	0.374	NEDD9	0.009	0.532
DDX20	0.002	0.375	INSIG1	0.009	0.533
SPSB1	0.009	0.377	ZBTB43	0.007	0.536
FUT1	0.009	0.377	DCP1A	0.006	0.536
FOXC1	0.003	0.382	CYTB	0.006	0.536
CENPBD1	0.002	0.384	PRR15L	0.007	0.540
DLX1	0.005	0.387	C9orf156	0.008	0.540
MIER2	0.003	0.388	USP35	0.008	0.540

ZNF555	0.002	0.389	BLOC1S3	0.006	0.540
ZNF200	0.002	0.390	CBX2	0.008	0.544
ZNF613	0.007	0.393	ABL1	0.008	0.545
C8orf58	0.002	0.393	THAP3	0.007	0.545
GATA6	0.004	0.395	ZNF503	0.010	0.546
CDC25A	0.002	0.398	ZNF107	0.008	0.551
SH3RF1	0.002	0.399	MEIS1	0.009	0.554
PF4	0.006	0.400	MED13L	0.008	0.558
C7orf40	0.003	0.401	SUZ12P	0.010	0.558
YRDC	0.004	0.401	SH3BP4	0.009	0.559
RPP38	0.002	0.402	C7orf49	0.010	0.559
PLEKHF2	0.010	0.402	MID1	0.009	0.559
CEBPB	0.006	0.403	GTF3C4	0.010	0.559
CRY2	0.006	0.404	A_33_P3317460	0.009	0.560
FADD	0.004	0.405	SETD1A	0.009	0.561
RNF19A	0.004	0.405	USP36	0.010	0.562
RLF	0.006	0.406	RSBN1L	0.008	0.563
ZFP36L1	0.010	0.407	AMD1	0.009	0.566
EPC2	0.003	0.407	TRAF2	0.008	0.568
IRS2	0.002	0.408	SHB	0.009	0.569
ZNF562	0.007	0.409	ZNF433	0.009	0.570
SMAD7	0.002	0.410	CCNJL	0.009	0.571
C1orf135	0.004	0.410	FAM110A	0.009	0.574
			MKL1	0.010	0.586

Functional clustering of significant genes was performed by the web-available program DAVID (Database for Annotation, Visualisation and Integrated Discovery) that clusters the genes into different categories such as tissue expression and function, according to the Gene-Ontology (GO) annotations (Dennis et al. 2003).

As seen in the table 11 functional clustering of down regulated mRNAs was performed using the DAVID gene ontology software. Among all 289 significantly down regulated mRNAs 97 were found to be involved in regulation of transcription. In addition, significant enrichment was demonstrated for ontology terms linked to apoptosis, cell proliferation, cell cycle, cell migration and angiogenesis. The 97 genes identified as being enriched in regulation of transcription included JUNB, MED9, MED13L, EGR1 and interleukin IL6. A transcription factor involved in cell migration, SNAI1, was absent from this category, which reflects defects in the annotation of genes in the ontology. We next validate the selected significantly expressed genes in the microarray by RT-qPCR.

Table 11: Functional clustering of 289 significantly down regulated genes:

Significantly downregulated genes were functionally clustered by microarray analysis in breast cancer cell line MCF-7 treated with cordycepin and identified using DAVID gene ontology analysis. Out of 289 downregulated genes 97 were found to be involved in transcriptional regulation followed by roles in cell proliferation and in cell migration (Done by MSc project student: Mangangcha Irengbam).

Functional cluster	No. of genes	Genes	*P* value
Regulation of transcription	97	*ATF3, ADRB2, ARID5B, PRR15L, BRF2, ABL1, CITED2, CEBPB, CD86, CBX2, CBX8, CRY2, CSRNP1, DDX20, DLX1, DLX2, E2F6, EGR1, EPC2, FOXA1, FOXC1, FOXL2, FOXQ1, , FOSL1, GATA6, GLIS2, HSF2, CENPBD1, ID1, ID3, ING1, IL11, IL6, JUNB, JUN, KLF5, KLF6, LIF, MED13L, MED9, MEIS1, MIER2, MIER3, MLL5, MYNN, NFIL3, NR4A3, PPRC1, LOC152845, PLAGL2, RHEBL1, RLF, RARA, RUNX1, SERTAD1, SERTAD2, SETD1A, SMAD6, SMAD7, TGIF1, TIGD5, TICAM1, TLE4, TRIB1, TRIM27, MAFF, MAFG, MYC, ZBTB3, ZBTB43, ZBTB45, BTB5, ZNF107, ZNF165, ZNF189, ZNF200, ZNF263, ZNF283, ZNF317, ZNF394, ZNF433, ZNF48, ZNF511, ZNF555, ZNF562, ZNF574, ZNF581, ZNF584, ZNF613, ZNF628, ZNF629, ZNF646, ZNF668, ZNF689, ZNF7, ZNF784, ZNF8, ZKSCAN2*	1.9E-19
Apoptosis	22	*AEN, AIFM2, C8ORF4, F3, CSRNP1, DDIT4, FADD, FOXL2, GATA6, IER3, IL6, JUN, NUAK2, PLEKHF1, PPP1R15A, RHOB, SGK1, SIAH1, SIAH2, SHB, TRAF2, MYC*	3.50E-04
Regulation of apoptosis	29	*ABL1, ADRB2, FOXL2, CEBPB, DLX1, DUSP1, F3, FOXC1, ID3, IL6, JUN, SMAD6, MYC, TRAF2, FOSL1, FADD, IER3, SOCS3, HERPUD1, CITED2, DDX20, CLCF1, SH3RF1, AEN, NUAK2, AIFM2, TICAM1, PIM3, LOC152845, PLAGL2*	3.50E-05
Angiogenesis	7	*CTGF, CYR6, ID1, JUN, KLF5, RHOB, SHB*	2.60E-02
Regulation of angiogenesis	5	*F3, ID1, IL6, RHOB, RUNX1*	1.50E-02
Regulation of cell proliferation	22	*ATF3, ADRB2, ADM, CD86, F3, FOSL1, HBEGF, ING1, IRS2, IL11, IL6, JUN, KLF5, LIF, PPM1D, SERTAD1, TGIF1, TICAM1, TOB2, TRIB1, MAFG, MYC*	8.80E-03
Cell cycle	21	*ABL1, CDC25A, CDT1, DUSP1, E2F6, HJURP, ING1, MLL5, PELO, PLK2, PLK3, PPP1R15A, PPM1D, RASSF1, SESN2, SIAH1, SIAH2, SH3BP4, MYC, WEE1, ZNF830*	1.50E-02
Regulation of cell cycle	17	*CITED2, CDC25A, CDT1, CYP1A1, FOXC1, FOSL1, ID3, JUNB, JUN, LIF, MAD2L1BP, PIM3, RHOB, SERTAD1, SMAD6, MYC, OBFC2A*	4.80E-05
Regulation of cell migration	7	*CITED2, F3, HBEGF, IRS2, IL6, SMAD7, TRIB1*	4.50E-02

6.2 Validation of selected candidates of microarray analysis

The use of P values in microarray analysis is a difficult subject. Because so many measurements are made, often a corrected P value is calculated, which allows more stringent testing. However, in the experience of our collaborators at the MRC toxicology unit in Leicester, this often excludes real changes. We therefore decided to estimate the false discovery rate experimentally. We performed qPCR on selected candidates that were predicted to be downregulated or unchanged from the microarray analysis. One upregulated mRNA (CD68) was included, as listed in table 12. The mRNA levels were expressed relative to GAPDH mRNA. In total 33 selected candidates from the microarray analysis proceeded for validation via RT-qPCR of which 6 show a discrepancy (shown in yellow) between RT-qPCR and

microarray analysis as seen in figure 6.1A and B. The rest of the validated mRNAs are shown in blue. All the bars of mRNAs below the red dotted line were downregulated by cordycepin as shown in bar graph figure 6.1 A. In figure 6.1 B, the scatter plot represents 2logFC qPCR and 2logFC microarray values on x axis and y axis respectively. The co-efficient of determination (R^2) was calculated which measures the percentage of the data closest to the regression line. The R^2 value of 0.29 indicates that 29% of data variance in y can be explicated through linear relationship between y and X while 71% of variance in y remains undetermined.

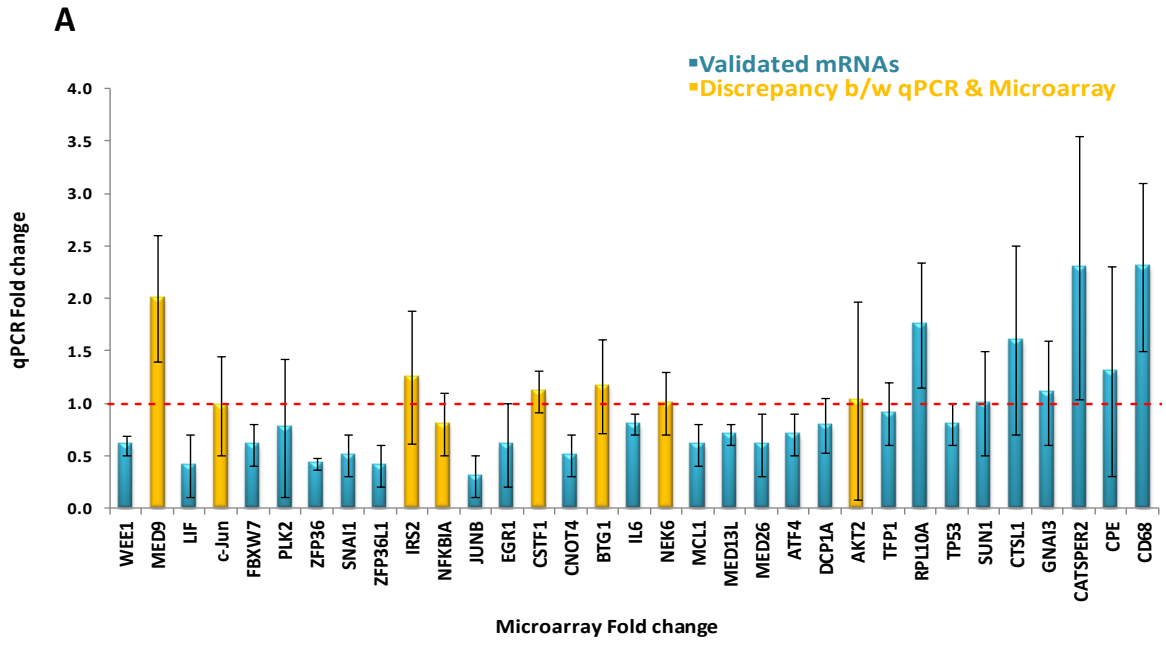
Table 12: List of selected validated mRNAs:

Fold change from RT-qPCR and fold change with P value from microarray data were shown for selected validated mRNAs. Standard deviation of the microarray data was calculated from the confidence interval (CI.025) by using POWER (2, (logFC-CI.025)). The table was categorized into downregulated (in pink), unchanged (in blue) and upregulated (in green).

mRNAs	qPCR		Microarray analysis	
	Fold change	Std. Dev	Fold change	P value
WEE1	0.60	0.09	0.228	0.002
MED9	2.00	0.60	0.229	0.004
LIF	0.40	0.30	0.266	0.002
c-Jun	0.98	0.47	0.269	0.001
FBXW7	0.60	0.20	0.279	0.003
PLK2	0.76	0.66	0.291	0.001
ZFP36	0.43	0.06	0.307	0.002
SNAI1	0.50	0.20	0.364	0.003
ZFP36L1	0.40	0.20	0.407	0.010
IRS2	1.25	0.64	0.408	0.002
NFKBIA	0.80	0.30	0.417	0.010
JUNB	0.30	0.20	0.417	0.003
EGR1	0.60	0.40	0.424	0.003
CSTF1	1.11	0.20	0.484	0.008
CNOT4	0.50	0.20	0.484	0.012
BTG1	1.16	0.45	0.490	0.010
IL6	0.80	0.10	0.531	0.008
NEK6	1.00	0.30	0.547	0.019
MCL1	0.60	0.20	0.547	0.019
MED13L	0.70	0.10	0.558	0.008
MED26	0.60	0.30	0.570	0.019
ATF4	0.70	0.20	0.665	0.027
DCP1A	0.79	0.26	0.837	0.270
AKT2	1.02	0.94	0.885	0.506
RPL10A	1.74	0.60	0.982	0.887
TP53	0.80	0.20	1.081	0.613
SUN1	1.00	0.50	1.123	0.602
TFPI	0.90	0.30	1.150	0.319
CTSL1	1.60	0.90	1.182	0.252
GNAI3	1.10	0.50	1.276	0.160
CATSPER2	2.29	1.26	1.348	0.070
CPE	1.30	1.00	1.526	0.033
CD68	2.30	0.80	1.810	0.021

Our data indicate that our method of analysis suggests that around 20% of mRNAs are differently classified in qPCR and microarray data. This is, when the P value cut-

off is relaxed to 0.02 for the downregulated mRNAs and a fold change of 0.66 is considered biologically relevant, while for upregulated mRNAs a fold change of 1.66 is used as a cut off. In fact, most of the discrepant mRNAs have low P values and a higher fold change. This apparent error rate is probably a combination of false discovery due to the large number of mRNAs tested and cases in which the qPCR and the microarray are not detecting the same mRNA, e.g. due to alternative splicing. Among all validated mRNAs four of the most cordycepin sensitive mRNAs (WEE1, MCL1, JUNB and SNAI1) were selected to examine its effects on PAP knockdown, adenosine analogues and stage on mRNA processing.



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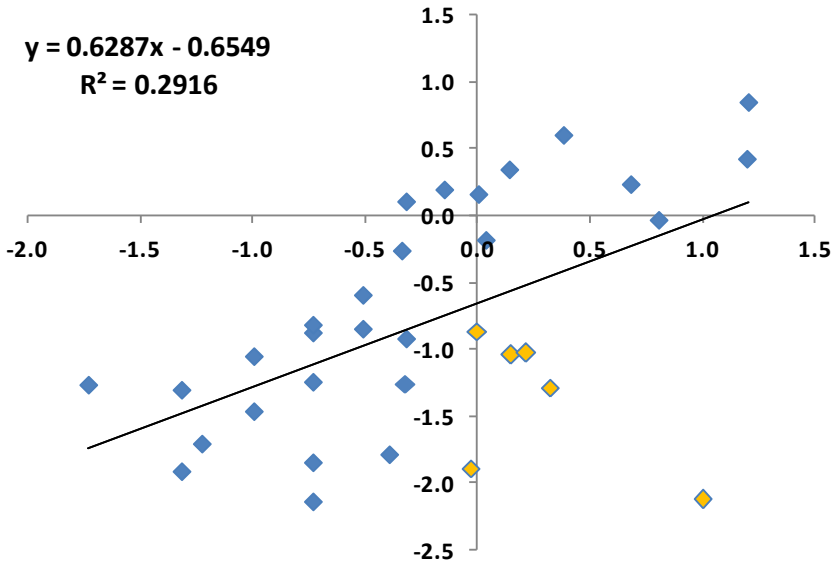

Figure 6.1: Validation of candidates from microarray analysis:

mRNAs were selected from microarray analysis on the basis of their real fold change and P values and proceed for validation via RT-qPCR. Total RNA was isolated from MCF-7 cells treated with cordycepin (50µM) for 2 hours. RT-qPCR was performed and the mRNA levels expressed relative to GAPDH mRNA. Among 33 tested mRNAs, 6 mRNAs shows discrepancy between RT-qPCR and microarray data represents in yellow bar while remaining 27 mRNAs in blue are validated. Control values of untreated (no cordycepin) were set at 1. Validation was performed on the same independent biological triplicates used for microarray analysis. **A,** Error bars show standard deviation over independent biological triplicates. Statistical significance compared to untreated (No cordycepin) were determined by paired T-test but none of them found to be statistically different from the untreated. **B,** Scatter plot showing 2logFCqPCR and 2logFCMicroarray on x axis and y axis respectively. Co efficient of determination R^2 was calculated as 0.2916.

6.3 The effect of adenosine analogues on four cordycepin targeted mRNAs

We next examined the effects of adenosine analogues on four of the cordycepin target mRNAs validated by RT-qPCR. MCF-7 cells were treated for 2 hours with indicated adenosine analogues at 50µM or as indicated, followed by total RNA isolation. RT-qPCR was performed and normalized to GAPDH. Similar effects were observed by cordycepin 50µM on all examined mRNAs except JUNB as shown in figure 6.2 for which greater sensitivity was observed. 8 aminoadenosine affects these mRNAs in a very similar manner to cordycepin. However 8 azidoadenosine

affected MCL1 only. Changes in levels of MCL1 were found to be statistically significant in the cordycepin and 8-azidoadenosine samples. For JUNB, cordycepin and 8 amino adenosine were found have statistically significant effects compared to untreated as shown in the figure 6.2.

It can be concluded that effects of adenosine analogues are also gene specific. Among all the mRNAs, JUNB is most affected by the polyadenylation inhibitors cordycepin and 8-aminoadenosine. Again, 8-aminoadenosine acts similarly to cordycepin.

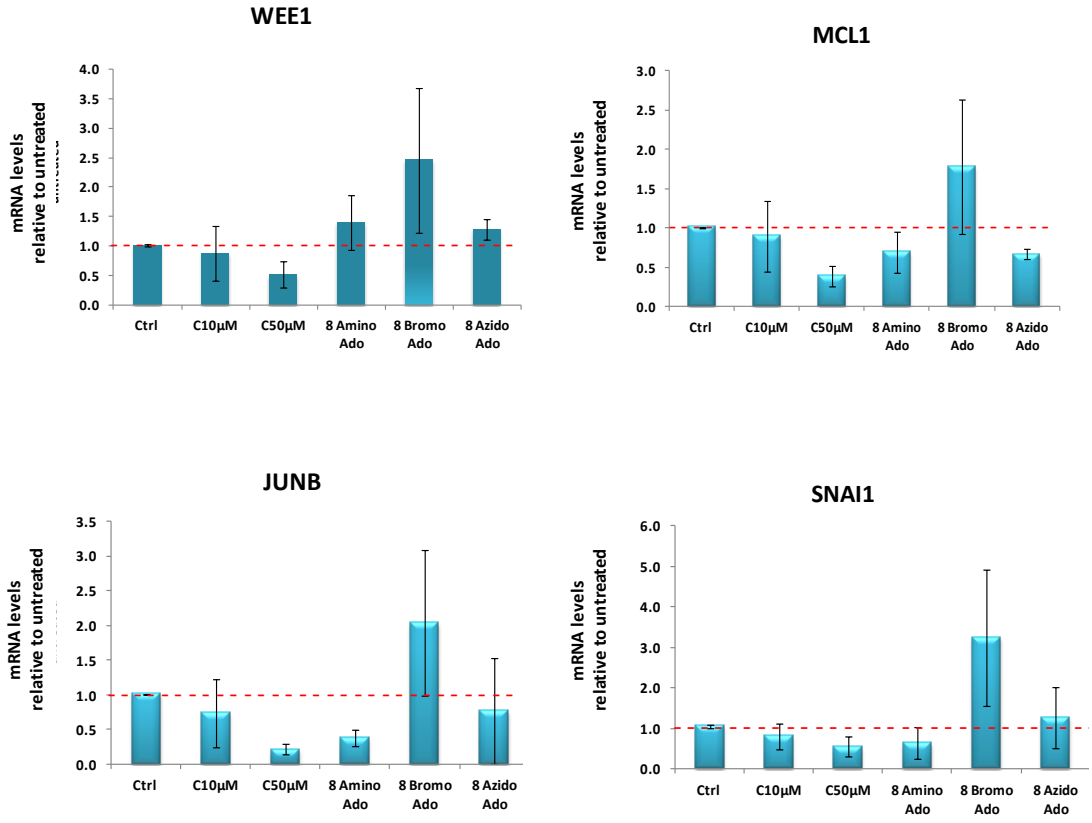

Figure 6.2: Effects of adenosine analogues treatment on selected mRNAs from microarray analysis:

Cordycepin treated most-sensitive mRNAs were selected from microarray analysis after validation via RT-qPCR. MCF-7 cells were treated with adenosine analogues (50µM) or as indicated for 2 hours. Total RNA was isolated and RT-qPCR was performed as described. The mRNA levels expressed are relative to GAPDH mRNA and control values of untreated (no cordycepin) were set at 1. Measurements were independent biological triplicates and data are expressed as means ±S.D. Statistical significance compared to untreated (No cordycepin) were determined by paired T-test but none of them were found to be statistically different from the untreated.

6.4 The effects of PAP knockdowns on four cordycepin targeted mRNAs

In order to investigate if specific PAPs are necessary for the expression of cordycepin target mRNAs we examined the effects on respective mRNAs by knock down experiments in MCF-7 cells. Cells were transfected twice, as described previously, and undergo total RNA isolation. RT-qPCR was performed and normalized to GAPDH. The untreated (sicontrol) control value is set as 1. It can be observed from figure 6.3 that none of the mRNAs were significantly affected by PAP knockdown.

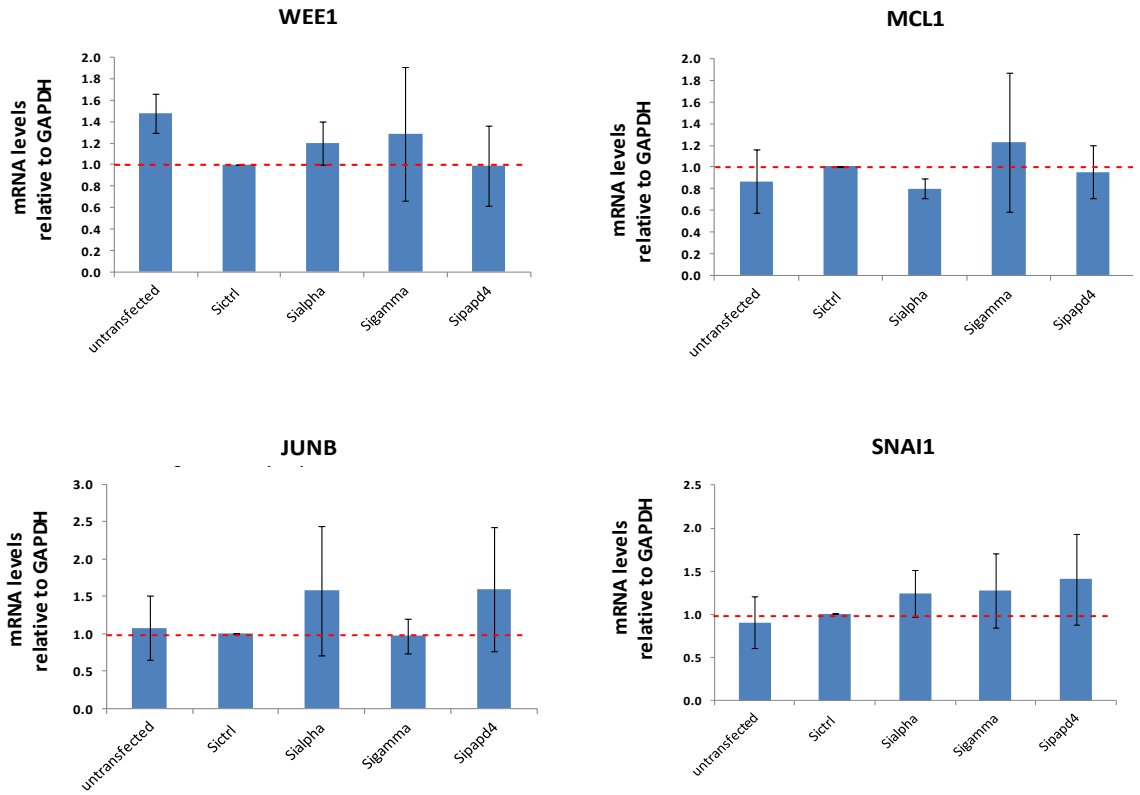

Figure 6.3: Effects of PAP knockdowns on selected mRNAs from microarray analysis:

Cordycepin targeted mRNAs were selected from microarray analysis after validation via RT-qPCR. MCF-7 cells were transfected twice with indicated siPAP for PAPOLA, PAPOLG and PAPD4 within 72 hours. Total RNA was isolated from MCF-7 and RT-qPCR was performed as described. The mRNA levels expressed relative to GAPDH mRNA and values for control siRNA were set at 1. Error bars shows standard deviations over independent biological triplicates. Statistical significance compared to sicontrol were determined by paired T-test but none of them found to be statistically different from the sicontrol.

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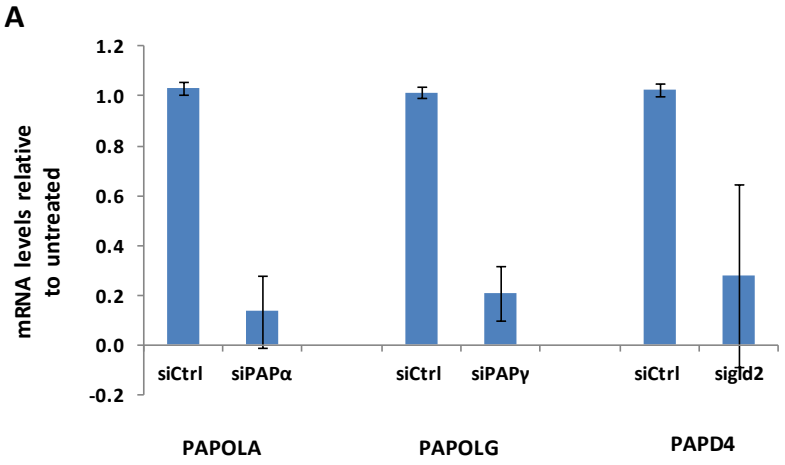
6.5 Combination PAP knockdowns does not affect JUNB mRNA expression

We next examined the effects of the combination PAP knockdowns on JUNB, because the combination knockdown was found to have a stronger effect on mRNA levels in the previous cordycepin and 8-aminoadenosine analogues sensitivity experiments. The MCF-7 cells were transfected with different combination of siRNAs against PAPs. As shown in figure 6.4A, knocked down reduced the levels of the corresponding PAP mRNAs. As can be seen in figure 6.4B, JUNB mRNA remains insensitive in the knock down experiments. It can be concluded that JunB expression is not dependent on these three PAPs and there is either redundancy with other PAPs or the residual expression of these PAPs after knockdown is sufficient for mRNA synthesis.



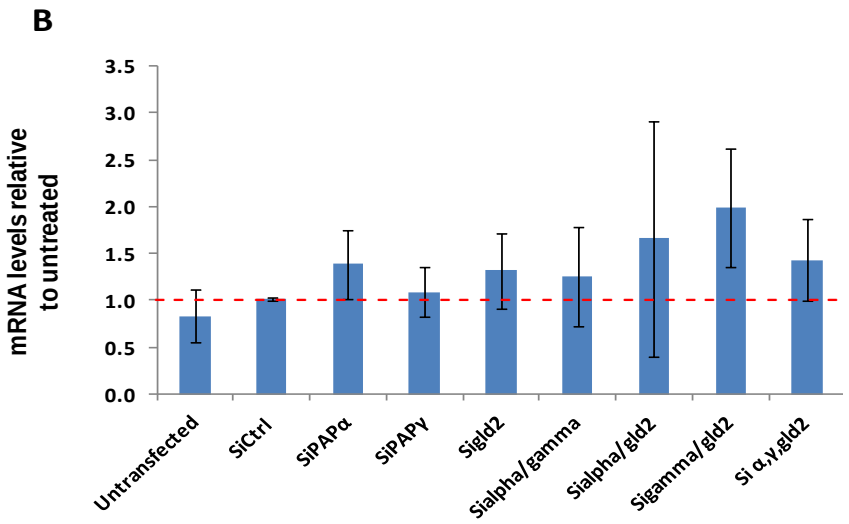


Figure 6.4: The effect of combination PAP knockdowns on JUNB expression:

MCF-7 cells were transfected twice in combination with indicated SiPAP for PAPOLA, PAPOLG and PAPD4 as described. mRNA isolation was done after 72 hours of first transfection and RT-qPCR for JUNB was performed as described. The mRNA levels expressed relative to GAPDH mRNA and values for control siRNA were set at 1. Error bar shows standard deviations over independent biological triplicates. **A,** knockdown qPCR was performed for siPAPOLA, siPAPLOG and siPAPD4 with relevant PAP primer. Statistical significance compared to sicontrol was determined by Paired T-test indicated as ** for P<0.01. **B,** qPCR for JUNB was performed on combination PAP knockdown samples. Statistical significance compared to sicontrol was determined by Paired T-test but none of the treatments caused a change in JUNB mRNA levels.

6.6 Post transcriptional effects of cordycepin on its target mRNAs

In order to examine at which stage of mRNA processing the effects of cordycepin occur, we next investigated the effect of cordycepin on mRNA level changes in cytoplasmic and nuclear fractions. As shown in figure 6.5 the mRNA levels in the cytoplasmic fraction were markedly reduced upon cordycepin treatment compared to the nuclear fraction. This indicates that cordycepin has a post-transcriptional effect on all four examined cordycepin target mRNAs.

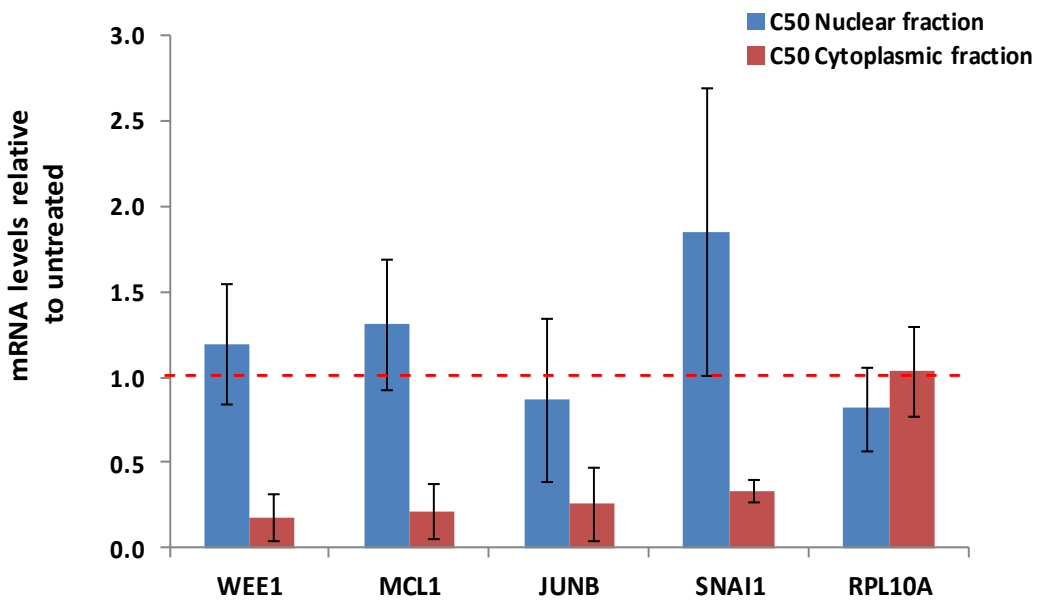


Figure 6.5: Post transcriptional effects of cordycepin:

Cordycepin treated sensitive mRNAs were selected from microarray analysis after validation via RT-qPCR. MCF-7 cells were treated with cordycepin (50μM) for 2 hours and nuclear and cytoplasmic fractions were separated as described. Total RNA was isolated followed by RT-qPCR for spliced mRNA. Four selected mRNAs were inhibited by cordycepin while RPL10A is insensitive. The mRNA levels are expressed relative to GAPDH mRNA and control values of untreated (no cordycepin) were set at 1. Error bars are standard deviations over independent biological triplicates. Statistical significance compared to untreated (No cordycepin) were determined by paired T-test but none of them found to be statistically different from the untreated.

6.7 Discussion

In this chapter we performed a genome wide study of gene profiling or microarray analysis in order to screen cordycepin sensitive mRNAs. From microarray analysis 289 significantly downregulated genes by cordycepin has been found, which cluster on a functional basis, determined by the web based gene ontology software DAVID. The statistical relevance of clustered gene associations was determined by DAVID, which uses a modified version of the Fischer Exact test in order to investigate the enrichment of grouped genes in comparison to the total number of genes assigned to that particular group in the human genome. From the DAVID gene ontology analysis it can be observed that out of 289, 97 mRNAs were involved in the regulation of transcription. In addition, there is an enrichment of genes with crucial role in regulation of cell proliferation, apoptosis, cell cycle, angiogenesis and

migration. These data indicate that cordycepin induces a reprogramming of cancer cells in order to induce cell death or differentiation.

We also found some genes involved in growth signalling and the small G-protein RHEBL1 (Ras homolog enriched in brain like 1 c), which is involved in promoting signal transduction via mTOR pathway (Tee et al. 2005). There are couple of significantly downregulated validated genes as found in figure 6.1 (A) which were not listed by DAVID such as SNAI1 (transcriptional repressor by downregulatig the expression of ectodermal genes within the mesoderm), MCL1 (a member of Bcnl-2 anti-apoptotic protein), FBXW7 (involves in phosphorylation-dependent ubiquitination) and CNOT4 (transcriptional regulator). This indicates that the annotation in the gene ontology database is inadequate. We proceeded to validate mRNAs from the microarray analysis via RT-qPCR and found among 33 examined mRNAs only 6 showed a discrepancy between RT-qPCR and microarray analysis. Among all examined adenosine analogues, only 8-aminoadenosine acts similarly to cordycepin by affecting all cordycepin target mRNAs, especially JUNB. However a PAP knockdown does not show any effects on the examined cordycepin target mRNAs. All four target mRNAs were post transcriptionally affected by cordycepin. It might be useful to investigate RHEBL1, as its downregulation by cordycepin might explain the effects of cordycepin on mTOR signalling.

7 Discussion and Conclusion

The main objective of this study is to characterize the effect of cordycepin on cellular proliferation, translation and gene expression. For this purpose several types of experiments were performed ranging from radioactive assays for the determination of rate of the protein synthesis and estimation of changes in total poly(A) tail length, to gene expression profiling. Several tested cell lines showed differences in their responses to cordycepin, but cordycepin inhibits cell proliferation in all cell lines as indicated in section 3. The variation in the cordycepin sensitivity of various cell lines may be due to differences in proliferation rates or in the levels of adenosine deaminase produced by the cells or present in the serum added to their media. The effects of cordycepin on protein synthesis and the levels of phosphorylated 4-EBP do not correlate with its effects on cell proliferation. The rate of cellular protein synthesis in cordycepin treated cells probably declines due to changes in translation initiation factors (Clemens 2004).

It has been observed previously in NIH3T3 cells that cordycepin down regulates the mTOR kinase pathway and up regulates AMP activated kinase (Wong et al. 2010). The effect of cordycepin was attributed primarily to the dephosphorylation of 4EBP. Through effects on mTORC2, cordycepin affected the activation of the protein kinase Akt, which is required for cell survival, adhesion and proliferation (Wong et al. 2010). Over expression or upregulation of AKT is involved in many cancers and the PI3K pathway in general is often upregulated in a variety of malignancies. Indeed, the MCF7 breast cancer cell line used in this study has an activating mutation in a PI3K subunit (Wu et al. 2005, Liang et al. 2006) (COSMIC database). Therefore, there is a lot of interest in adenosine related drugs such as ATP competitive inhibitors for PI3K/AKT pathway or CDK inhibitors involved in targeting cell cycle kinases (Lindsley et al. 2008, Galons et al. 2010, Lindsley 2010). Because ATP is required in many biological functions it might be possible that these drugs have multiple targets and some unknown targets result in toxicity of these drugs. Cordycepin, as a known inhibitor of the mTORC pathways, was therefore thought to be a good candidate for a novel type of cancer drug. Similarly, as a close adenosine analogue, cordycepin could interfere in a myriad of cellular processes, which

require adenosine or its nucleotides (Lee et al. 2012). However, a possible explanation is that in most situations, cordycepin triphosphate is either not recognized as a substrate by the relevant enzymes or that it is used in place of normal ATP as it contains the phosphate groups which could contribute normally to for instance, phosphorylation reactions. The cell type specific effects on protein synthesis and signal transduction indicate that not all effects of cordycepin are the same for all cell types. However, since all cell lines tested are inhibited in growth and show effects on polyadenylation, it is possible that the primary effects of cordycepin are more gene specific than cell type specific. One possibility is that cordycepin primarily affects rapidly transcribed genes, for instance because they need efficient transcription termination. As not all cell types transcribe the same genes, the effects would naturally be variable. This possibility is currently being tested in the De Moor laboratory. My work confirms that cordycepin affects the expression of specific mRNAs, including IL8, CXCL1, TWIST1 and the mRNAs identified in the microarray, which include JUNB, SNAI1, WEE1 and MCL1. The functional analysis does indeed indicate that the inhibited mRNAs are linked to cell proliferation, survival and migration. In addition, the high number of transcription factors shows that cordycepin is likely to cause secondary changes in gene expression in the longer term.

Due to rapid clearance of cordycepin from the medium exposure is usually quite brief. Thus it has been administered with another potential drug such as pentostatin, an adenosine deaminase inhibitor, to increase its efficacy over longer time points (Tsai et al. 2010). An interesting observation detected in the present study is that a minimal dose of cordycepin and pentostatin together, increases its effectiveness by successfully stabilizing cordycepin over longer time points. This reduces the rate of protein synthesis and increases anti-proliferative activity. Thus degradation of cordycepin is also a factor in tissue culture experiments. Therefore pentostatin serves as a stabilising agent especially at longer time points. Indeed, pentostatin and another purine related analogue, cladribine, has been used in B-chronic lymphocytic leukemia (Kay et al. 2007). My work indicates that lower doses of pentostatin may still be effective.

Consistent with the previously published data in ASM cells (Kondrashov et al; 2012) 8-aminoadenosine affects polyadenylation in NIH3T3 to greater extent compared to MCF-7 cells. From section 5 it has been seen that in HELA 8-aminoadenosine effects similar to cordycepin by inhibiting the rate of protein synthesis through a decline in levels of P-4EBP. In MCF-7 cells it also acts similar to cordycepin by inhibiting the rate of protein synthesis, polyadenylation and cell proliferation. In most cases, 8-aminoadenosine also affects gene expression in the same way as cordycepin. This is remarkable, since despite having their modifications in completely different parts of the adenosine moiety, both cordycepin and 8-aminoadenosine cause chain termination of polyadenylation (Zarkower and Wickens 1987, Chen et al. 2010), rather than inhibiting polyadenylation in any other way. This appears to indicate that the arrest of the cleavage complex on prematurely terminated poly(A) tails may play a large role in the mechanism of action of cordycepin.

Like cordycepin, 8-aminoadenosine has been reported to mediate inhibition of the PI3K/AKT/mTOR and MAPK signaling pathway by decreasing the levels of p-AKT (Ghias et al. 2005), P-4EBP and p-ERK1/2 respectively (Dennison et al. 2010). It may also lead to inhibition of RNA transcription (Frey and Gandhi 2010). 8-amino adenosine has been shown to induce autophagic cell death (Shanmugam et al. 2009) induce apoptosis and inhibits cell proliferation in MCF-7 cells (wild type p53) and T47-D cells (mutant p53) and estrogen receptor negative MDA-MB-231 or MDA-MB-468 (mutant p53) breast cancer cell lines (Polotskaia et al. 2012). Previously it has been shown that 8-aminoadenosine is highly cytotoxic to myeloma cancerous cells compared to normal lymphocytes (Krett et al. 2004, Dennison et al. 2010). These data show that others also find that cordycepin and 8-amino adenosine are compounds with similar potentially therapeutic properties.

In MCF-7 cells, cell proliferation is affected by 8-azidoadenosine and cladribine while DRB and 8-bromoadenosine affect cell proliferation to a lesser extent. It could be interesting to explore other cell proliferation and cell cycle assays to elucidate the mode of action of these analogues, as they all appear to act differently. Among the examined adenosine analogues cladribine is known to interfere with DNA synthesis and RNA transcription while Metformin is an AMPK

activator used in type II diabetes and has been reported to have similar cellular effects to cordycepin (Zakikhani et al. 2008, Martin-Castillo et al. 2010). DRB does not affect translation or the levels of P-4EBP in NIH3T3 cells (Fig 5.2), despite affecting polyadenylation (data not shown). DRB is known to affect polyadenylation by reducing the recruitment of polyadenylation factors to RNA polymerase II (Glover-Cutter et al. 2008). Like DRB, Se13 and Se15 are novel, highly specific CDK9 inhibitors, but they do not inhibit polyadenylation. It indicates the possibility that different cdk-like kinases are involved in this process. A likely candidate is CKD12, which is sensitive to DRB, but perhaps not to Se13 and Se15. CDK12 was recently shown to be the major RNA pol II kinase involved in 3' mRNA processing (Davidson et al., 2014).

I observed that all three PAPs I examined are necessary for cell proliferation while knocking down of these PAPs does not affect the bulk polyadenylation in MCF-7 cells. In airway smooth muscle cells, knockdown of PAPOLA affects the induction of inflammatory mRNAs (Kondrashov et al. 2012), but we did not observe effects of PAP knockdown on steady state mRNA levels for any of the cordycepin sensitive mRNAs. The sensitivity of inflammatory mRNAs (CXCL1 and IL8) in MCF-7 cells to 8-aminoadenosine and cordycepin was as reported previously in ASM cells (Kondrashov et al. 2012).

In contrast to the observations in airway smooth muscle cells, where some cordycepin sensitive mRNAs were affected post-transcriptionally and others at the transcriptional level (Kondrashov et al., 2012), all cordycepin sensitive mRNAs that I examined in MCF7 cells were affected primarily at the post-transcriptional level. This is a strong indication that the effect of cordycepin on these genes is through its effect on polyadenylation, rather than on signal transduction, which would be expected to act primarily by affecting transcription. Given that so many transcription factor mRNAs are affected by cordycepin, one could expect secondary effects on transcription to follow soon.

The microarray analysis discussed in section 6 provides wider a platform for the screening of cordycepin sensitive mRNAs in MCF-7 cells. Functional clustering of

down regulated mRNAs by DAVID gene ontology software reveals a high percentage of gene involvement in transcriptional regulation and apoptosis followed by cell proliferation, cell cycle and cell migration to angiogenesis. This implies the possible role of cordycepin in the attenuation of ERK signaling pathway in two ways. Firstly as the majority of downstream targets regulated by the ERK pathway are mostly affected by cordycepin such as transcription regulators (JunB, EGR1, MED13L, MED26, ZFP36, SNAI1, ZFP36L1, NFKBIA, FBXW7), cell proliferation regulators (LIF, ATF3, TOB2, MYC), cell cycle regulators (WEE1, DUSP1, PLK2), cell migration regulators (IL6) and regulators of angiogenesis (RUNX1). Secondly, AMPK activation has been previously reported upon cordycepin treatment (Wong et al. 2010), which leads to downregulation of BRAF through phoshorylation and inhibition of the ERK signaling cascade (Shen et al. 2013). It would therefore be important to examine the effect of cordycepin on the ERK signaling cascade in MCF7 cells. The fact that all cordycepin sensitive mRNAs (WEE1, MCL1, JUNB, SNAI1, IL8, CXCL1) are not affected by PAP knockdown, despite the post-transcriptional effect indicates that cordycepin does perhaps not work by inactivating poly(A) polymerases, but that the arrest of the cleavage complex may be the crucial mechanism. It will be important to investigate the formation of the cleavage complex *in vivo* and to develop methods for arresting it independent of chain termination, for instance by the expression of inactivated poly(A) polymerases.

Interestingly, the cytoplasmic polyadenylation factor CPEB4 has been shown to promote malignant transformation through inducing the EMT by positively regulating mesenchymal markers (such as SNAI1) (Xu and Liu 2013). By examining some other important mRNAs, such as RHEBL1 found in microarray analysis, will be useful in order to clarify the effects of cordycepin on the mTOR pathway. Our microarray analysis strongly indicates the potential of cordycepin as an anticancer drug as it has direct effects on key mRNAs involved in regulating cell proliferation, migration and survival.

Proposed future work includes investigation of the effect on cell cycle specific stages of cordycepin and PAP knockdown using FACS analysis and transfection with mutant PAPs. Also, to investigate if the effect of cordycepin on Akt phosphorylation

is the major cause of its anti-proliferative effect, it will be useful to investigate if expression of a constitutively active Akt mutant mediates cordycepin resistance of signal transduction and cell proliferation. It will be necessary to investigate the effects of PAP knockdown and overexpression on signal transduction to test the hypothesis that the inhibition of polyadenylation is causing the effects of cordycepin on signal transduction. Polysome profiling of the effects of cordycepin on mRNA translation (Bushell et al. 2006) or poly(A) fractionation (Meijer et al. 2007) may identify mRNAs with shortened poly(A) tails that are not reduced in abundance, but in translational efficiency.

Taken together, my data indicate that cordycepin is a potential lead chemotherapeutic agent for the treatment of breast cancers and the main pathway is likely to be through effects on polyadenylation. This study provides some new insights into the mechanism of action of cordycepin, which will provide better understanding by enhancing the knowledge of the control of gene regulation of breast cancer cells, with consequences for the development of different cordycepin derivatives for breast cancer therapies.

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